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PRINCIPAL INVESTIGATOR: Xuesong Ouyang, Ph.D.

CONTRACTING ORGANIZATION: University of Medicine and Dentistry of New Jersey
Piscataway, New Jersey 08854-5635

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Xuesong Ouyang, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Medicine and Dentistry of New Jersey
Piscataway, New Jersey 08854-5635

*E-Mail:* ouyang@cabm.rutgers.edu

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**13. ABSTRACT (Maximum 200 Words)**

*Nkx3.1* is a prostatic-specific tumor suppressor whose loss-of-function represents a critical step in prostate cancer initiation. However, the molecular basis is still largely unknown. We have been utilizing microarray analysis to pursue the gene expression profiling of prostatic lesions in the *Nkx3.1* mutant mouse model relative to normal prostate epithelium. Our findings suggest that *Nkx3.1* loss-of-function leads to deregulated secretory function of prostate which representing a defect differentiation of prostate epithelium, thus may contribute to the increased susceptible to carcinogenesis. Moreover, *Nkx3.1* mutant prostates are deficient for anti-oxidative protection as a consequence of aging, which underlies its role in cancer predisposition. In an effort to explore the molecular alterations in advanced stages of prostate cancer progression, we find that the mutant mice share many features in common with human prostate cancer, including over-expression of various putative markers for human prostate cancer (*e.g., Ezh2, Pim1, Hepsin, and Clusterin*). Furthermore, up-regulation of *AP-1* components in androgen-independent tumor cells, as well as activation of *cyclin D1* in the *Nkx3.1;Pten;p27* compound heterozygous tumor cells, may reflect novel molecular pathways underlying prostate cancer progression.

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## Table of Contents

## I. Training accomplishments

During the funding period of March 2003 through February 2005, I have received extensive training under the guidance of the supervisor, Dr. Cory Abate-Shen, as I have made considerable progress toward identifying *Nkx3.1*-responsive genes involved in prostate carcinogenesis. With this guidance and training, I have established and optimized the methodologies needed for this study, including laser capture microdissection, RNA isolation and amplification, Affymetrix microarray and data analysis, real-time PCR, *in situ* hybridization and immunohistochemistry. With the help of Whitney Banach-Petrosky, a senior technician of our laboratory, I have also set up tissue/cell recombination and orthotopic injection assays for functional study of candidate target genes of *Nkx3.1* during prostate carcinogenesis.

In addition to the technical support, intellectually, my study has also benefited from the guidance and assistance of Dr. Abate-Shen and other laboratory members. On a weekly basis, I discuss the research project in detail with Dr. Abate-Shen; and on a bi-weekly basis, I present the data at the laboratory meetings. These discussions of the progress and pitfalls of my research project have promoted critical evaluation of the data on a consistent basis. Moreover, many research investigators at CABM and Cancer Institute of New Jersey have provided valuable advices on my research project in many aspects. These include our collaborators, Dr. Michael Shen, who has extensive expertise in prostate biology; and Drs. Yong Lin and Weichung J. Shih, who have provided considerable assistance in microarray data analysis.

Finally, I had the opportunities to attend several symposiums/retreats at CABM, Cancer Institute, and the UMDNJ-Rutger's life science community during last funding period. I presented two abstracts (one on myself and one as a coauthor) at the 95$^{th}$ annual meeting of American Association for Cancer Research in March 2004. I will also be attending the Genome Access Course at Cold Spring Harbor Laboratory in April 2005.

## II. Research accomplishments

### A. Introduction

Our **hypothesis** is that *Nkx3.1* homeobox gene functions as a transcription factor to regulate gene expression during normal prostate growth and differentiation, and that loss of *Nkx3.1* leads to the aberrant expression of target genes that ultimately contribute to prostate carcinogenesis. Therefore, identification of *Nkx3.1* target genes will provide insight into the molecular pathways associated with prostate carcinoma.

We have demonstrated that the *Nkx3.1* mutant mice display histopathological features of prostatic intraepithelial neoplasia (PIN), the presumed precursor of human prostate cancer, which increase in severity with age [1, 2]. Furthermore, prostatic-specific loss-of-function of *Nkx3.1* cooperates with loss-of-function of broad-spectrum tumor suppressors such as *Pten* and/or $p27^{kip1}$ in cancer progression [3, 4].

Using Affymetrix GeneChip expression profiling, as validated by real-time PCR and immunohistochemistry, we have now found that loss-of-function of *Nkx3.1* in mice leads to a deregulated secretory function in prostate, which represents a less differentiated secretory tissue and may contribute to increased susceptibility to prostate carcinogenesis. Furthermore, our findings suggest *Nkx3.1* loss-of-function leads to accumulation of oxidative damage in prostate as a consequence of aging, which underlies its role in cancer predisposition [5].

To explore *Nkx3.1*-responsive genes during more advanced stages of prostate cancer progression, and to verify the synergistic activity of *Nkx3.1*, *Pten* and/or $p27^{kip1}$, *Nkx3.1;Pten* and *Nkx3.1;Pten;p27* compound mutant prostates were analyzed by Affymetrix microarray accompanied with the laser capture microdissection (LCM) technique. Our findings have revealed that the mutant mice share many features in common with human prostate cancer, including over-expression of various putative markers for human prostate cancer (*e.g., Ezh2, Pim1, Hepsin, and Clusterin*). Furthermore, up-regulation of *AP-1* components in androgen-independent tumor cells, as well as activation of *cyclin D1* in the *Nkx3.1;Pten;p27* compound heterozygous tumor cells, may reflect novel molecular pathways underlying prostate cancer progression.

**B. Body** Below we list our goals for the last funding period (from SOW) and a description of the status.

**Task 1. Isolation of *Nkx3.1*-responsive genes by microarray analysis.**

This goal has been successfully implemented. Microarray analyses were performed using mRNA from prostates (anterior and dorsolateral lobes) of *Nkx3.1* homozygous mutant and wild-type mice at 15-month-old of age. As visualized by two-way hierarchical clustering, a total of 638 genes were differentially expressed following *Nkx3.1* loss-of-function. Among them are deregulated expression of 4 genes encoding seminal vesicle secretory proteins and prostatic specific probasin (Fig. 1). Moreover, we have uncovered an intriguing link between loss-of-function of *Nkx3.1* and loss of protection against oxidative damage (Fig. 2 and [5]). *We propose that the essential role of Nkx3.1 in maintaining the terminally differentiated state of the prostate*

*epithelium provides protection against oxidative damage and, thereby, suppression of prostate cancer.* Validations of microarray findings have been done as described in summary of Task 4.

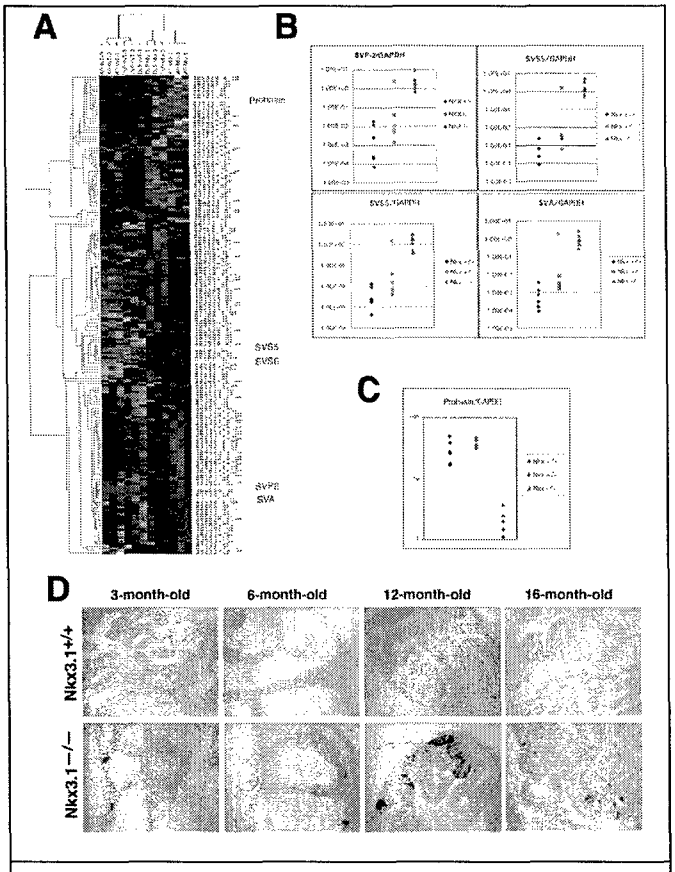


**Figure 1. Deregulated secretory function of *Nkx3.1* mutant prostates.** (A) Gene expression profiling comparing *Nkx3.1* wild-type and mutant prostates (dosalateral and anterior lobes). Among a total of 638 deregulated genes are genes encoding seminal vesicle or prostate secretory proteins, including *SVP2*, *SVS5&6*, *SVA* and *probasin*. (B) Real-time RT-PCR analyses of mRNA levels of seminal vesicle secretory proteins. (C) Real-time RT-PCR analysis of probasin. (D) Immunohistochemical analysis of SVP2 in anterior prostates from *Nkx3.1* wild-type or mutant mice of different ages.

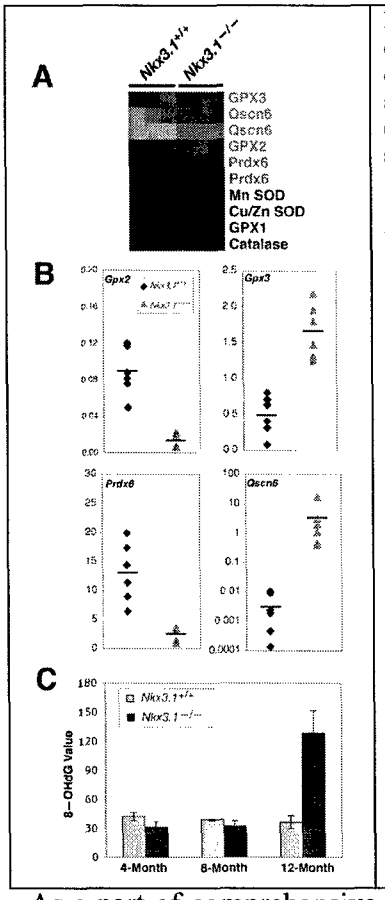


**Figure 2. Loss-of-function of *Nkx3.1* results in deregulation of protection against oxidative damage.** (A) Hierarchical clustering of selected gene expression differences between the anterior prostates of wild-type ($Nkx3.1^{+/+}$) and *Nkx3.1* mutant ($Nkx3.1^{-/-}$) mice. (B) Real-time PCR analysis showing the relative expression levels of *GPx2*, *GPx3*, *Prdx6*, and *Qscn6* (as indicated) in anterior prostates from wild-type (blue) and *Nkx3.1* mutant (orange) mice. The bars indicate average value of 6 samples. (C) Relative levels of 8-OHdG in genomic DNA obtained from age-matched wild-type (light grey) and *Nkx3.1* mutant (dark grey) mice. The data are expressed as value of 8-OHdG measured by LC-MS/MS. Note that the older *Nkx3.1* mutant mice display increased 8-OHdG values. Error bars indicate the standard errors of 3 samples.

As a part of comprehensive molecular profiling of prostate cancer progression in *Nkx3.1; Pten* mutant mice, we have also performed microarray analyses of LCM samples using 4 prostatic lesions (low-grade PIN) from *Nkx3.1* mutant mice and 4 normal prostatic epitheliums of age-matched wild-type mice. See summary of Task 2 for details.

However, the comparison of prostatic lesions *vs.* adjacent normal prostatic epithelium of $Nkx3.1^{-/-}$ mice is technically difficult since the heterogeneity and multifocality of the lesions in *Nkx3.1* mutant prostate made them hard to be captured from cryosections. Instead, comparison of lesions and normal prostatic epitheliums was performed using different samples.

**Task 2. Isolation of target genes for synergistic activity of *Nkx3.1* and *Pten* loss-of-function by microarray analysis.**

Since *Nkx3.1; Pten* mutants recapitulate many stages of prostate cancer, they provide a unique opportunity to investigate molecular changes associated with prostate carcinogenesis. We had used Affymetrix GeneChip microarrays to compare the gene expression patterns of prostatic epithelium from each of the following: *(i)* **benign/normal** glands from wild-type mice (age- and strain-matched with mutants); *(ii)* **low-grade PIN** from *Nkx3.1* mutants at 12 months; *(iii)* **high-grade PIN** from *Nkx3.1; Pten* compound mutants at 6-9 months; *(iv)* **adenocarcinoma** from *Nkx3.1*; *Pten* compound mutants at 12-15 months; *(v)* **androgen-independent high-grade PIN** from *Nkx3.1; Pten* compound mutants castrated at 6 months and analyzed at 9 months; *(vi)* **androgen-independent adenocarcinoma** from *Nkx3.1; Pten* compound mutants castrated at 12 months and analyzed at 15 months. Some of these data were published in [4] and [6]. However, we have not further pursued the comparison of PIN lesions *vs.* adjacent normal epitheliums *Nkx3.1;Pten* compound mutants because of the similar aforementioned technical impractibility.

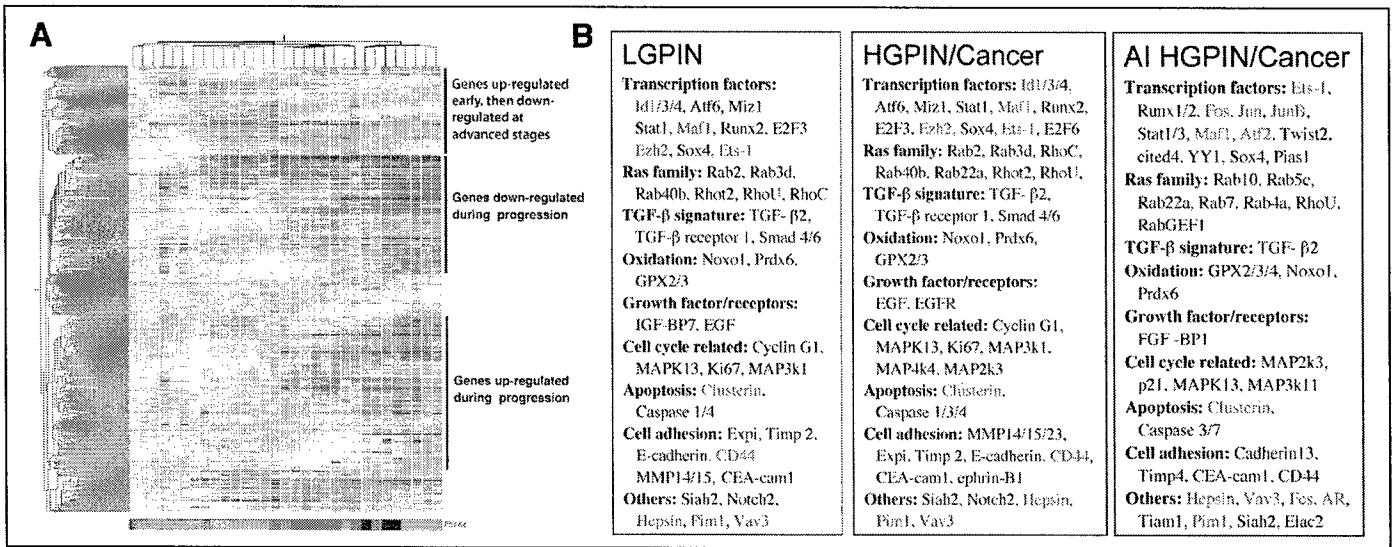


**Figure 3. Gene expression profiling of *Nkx3.1; Pten* mutant mice at various stages of prostate carcinogenesis.** (A) Hierarchical cluster analyses of 554 deregulated genes in prostate cancer progression. (B) Deregulated genes/pathways of particular interest. Note that *Nkx3.1; Pten* mutant mice display several gene expression changes known to be occur in human cancer including *clusterin, hepsin, Pim1 and Ezh2* (red). Also note that androgen-independent tumors exhibit significant up-regulation of major AP-1 components, including *c-For, c-Jun, Atf2 and Mafl* (red).

As shown in Fig. 3, we have identified clusters of genes that are progressively lost or activated at discrete stages during cancer progression based on the statistical, as well as biological significance. These include a number of genes/pathways that known to be deregulated in human prostate cancer, such as components of the Ras signaling pathway, oxidative damage pathways, proliferation/ apoptosis pathways and others (Fig. 3B). In particular, we find that several genes, which have been previously demonstrated to be markers for **human** prostate cancer (*e.g., Ezh2, Pim1, Hepsin, Clusterin*), are also up-regulated during cancer progression in the mutant mice. *This provides further evidence that the molecular features of prostate carcinogenesis are at least partially conserved between humans and the mutant mice.* Furthermore, we have identified several genes strikingly altered in androgen-independent prostate cancer, including four major *AP-1* transcription factor components: *c-Fos, c-Jun, Atf2, and Mafl* (Fig. 3B). Currently, we are at various stages of validating the expression status of AP-1 components to mouse and human prostate cancer.

**Task 3. Exploration of *Nkx3.1* regulated genes in the development of PIN to prostate cancer.**

We proposed to explore *Nkx3.1*-responsive genes in advanced stages of prostate carcinogenesis using a tissue recombination approach. However, our recent studies have shown that *Nkx3.1;Pten* and *Nkx3.1;Pten;p27$^{kip1}$* compound mutants recapitulate various stages of prostate carcinogenesis from low-grade PIN to high-grade PIN, to invasive adenocarcinoma, and metastases to the distant tissues [3, 4, 7]. Therefore, we have not pursued the tissue recombination approach. Instead, by using the double (*Nkx3.1;Pten*) and triple (*Nkx3.1;Pten;p27$^{kip1}$*) compound mutants, we extended our proposed task 3 not only to explore *Nkx3.1*-responsive genes, but also to explore the cooperativity of prostate-specific tumor suppressor *Nkx3.1* and broad-spectrum tumor suppressors such as *Pten* and *p27$^{kip1}$* during multi-stages of prostate cancer progression.

For this purpose, we have performed gene expression profiling using LCM samples of 9 double (*Nkx3.1;Pten*) mutants and 7 triple (*Nkx3.1;Pten;p27$^{kip1}$*) mutants, which representing the progression of prostate cancer from PIN lesions to invasive adenocarcinoma. Our microarray data analyses of triple mutants have revealed that *Nkx3.1* loss-of-function cooperates with heterozygosity of *p27$^{kip1}$* in promotion of prostate carcinogenesis through up-regulation of *cyclin D1* (Fig. 4 and [4]).

**Task 4. Validation of *Nkx3.1*-responsive genes.**

As mentioned in summary of Task 1, microarray analyses of *Nkx3.1* mutant and wild-type prostates have revealed a robust increase in expression of 4 genes encoding secretory proteins that are normally expressed by the seminal vesicle (Fig. 1A). These findings have been further confirmed by real-time PCR (Fig. 1B&C) and immunohistochemical studies (Fig. 1D). We thus propose that *Nkx3.1* is required for prostatic epithelial specification; in its absence the prostatic epithelial of *Nkx3.1* mutants represents defects in differentiation which may contribute to the increased susceptible to carcinogenesis. To test this hypothesis, we have developed two immortalized cell lines from embryonic urogenital epithelium (UGE) or seminal vesicle epithelium (SVE) for functional study of *Nkx3.1* using a tissue/cell recombination approach. We are using the

*Nkx3.1* mutants and UGE/SVE cells as models to study the consequences of aberrant differentiation for cancer susceptibility in prostate.

Furthermore, gene expression profiling, as validated by real-time PCR, have revealed a significant reduction in expression of glutathione peroxidase 2 (*GPx2*) and peroxiredoxin 6 (*Prdx6*), and up-regulation of glutathione peroxidase 3 (*GPx3*) and sulfyhydryl oxidase Q6 (*Qscn6*) (Fig. 2A & B), which consequently led to accumulation of oxidative damage of DNA in prostates of aged (*i.e.,* 12 months) mutant mice, as evident by significantly increased levels of 8-OHdG (Fig. 2C). Our findings support the idea that reduced protection against oxidative damage is an early event in prostate carcinogenesis, and suggest that loss-of-function of *Nkx3.1*, which occurs frequently at early disease stages, may explain, at least in part, why the prostatic epithelium is so vulnerable to oxidative stress. We envision that *Nkx3.1* mutant mice will be valuable for studying the relationship between the oxidative damage response and carcinogenesis, as well for pre-clinical studies to test the efficacy of potential anti-oxidants for cancer prevention.

## C. Key research accomplishments

- Gene expression profiling of *Nkx3.1* wild-type and mutant prostates reveal a total of 638 genes are differentially expressed following *Nkx3.1* loss-of-function;
- Deregulated secretory function in *Nkx3.1* mutant prostate reflects a defective differentiation of prostate which may contribute to the increased susceptible to carcinogensis;
- Loss-of-function of *Nkx3.1* leads to accumulation of oxidative damage in prostate;
- Comprehensive molecular profiling analyses have revealed that the *Nkx3.1; Pten* mutant mice share many features in common with human prostate cancer, including over-expression of various putative markers for human prostate cancer (*e.g., Ezh2, Pim1, Hepsin, and Clusterin*). Furthermore, our findings of a close association between significant up-regulation of *AP-1* components and androgen-independent prostate cancer indicate that up-regulation/activation of AP-1 transcription factors represent a novel pathway in the transition to androgen-independence;
- *Nkx3.1* cooperates with heterozygosity of $p27^{kip1}$ in promotion of prostate carcinogenesis through up-regulation of *cyclin D1*.

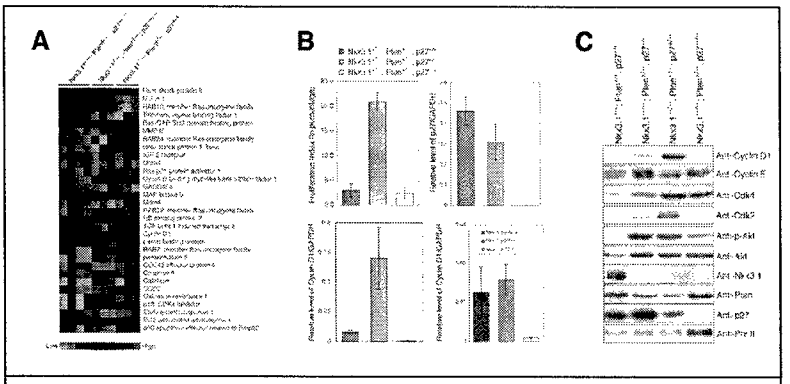


**Figure 4. Up-regulation of cyclin D1 in compound heterozygotes.** (A) Gene expression profiling comparing compound mutant mice having two, one, or no alleles of $p27^{kip1}$. RNA was obtained by LCM from dorsolateral prostate to isolate prostate PIN or cancer cells. Selected cancer related genes are shown. Among those is cyclin D1. (B) Expression analyses. Quantitation of the proliferation of prostate epithelial cells in PIN and cancer lesions from compound mutant mice (*upper left*); Real-time RT-PCR analyses of $p27^{kip1}$ (*upper right*) and *cyclin D1* (*bottom left*) in LCM isolated prostatic PIN or cancer cells from compound mutant mice; Real-time RT-PCR analyses of *cyclin D1* in LCM isolated prostatic epithelium from *Nkx3.1*;$p27^{kip1}$ double compound mice (*bottom right*). (C) Western blot analyses. Whole cell extracts were made from anterior prostate of mice of the indicated genotypes. Pol II detects the large subunit of RNA polymerase II is an internal control for protein loading.

## D. Reportable outcomes

- **Abstracts**
  1. **Ouyang, X.**, DeWeese, T.L., Nelson, W.G., Abate-Shen, C. (2004) Loss of function of Nkx3.1 predisposes to prostate cancer through increased oxidative damage as a consequence of aging. *Pro. Am. Assoc. Cancer Res.* 45: No. 770.
  2. Gao, H., **Ouyang, X.,** Banach-Petrosky, W., Kim, M., Lee, H., Sun, X., Borowsky, A., Cardiff, R., Lee, Y., Shih, W.J., Shen, M., and Abate-Shen, C. (2004) Heterozygosity of $p27^{kip1}$ promotes prostate carcinogenesis through up-regulation of cyclin D1. *Pro. Am. Assoc. Cancer Res.* 45: No. 5560.
- **Manuscripts**
  1. Gao, H., ***Ouyang, X.,** Banach-Petrosky, W., Borowsky, A.D., Lin, Y., Kim, M., Lee, H., Sun, X., Shih, W.J., Cardiff, R.D., Shen, M.M., Abate-Shen, C. (2004). A critical role for $p27^{kip1}$ gene dosage in a mouse model of prostate carcinogenesis. *Proc Natl Acad Sci USA* 101:17204-9. **(*co-first author)**
  2. Kuzmichev, A., Margueron, R., Vaquero, A., Preissner, T.S., Scher, M., Kirmizis, A., **Ouyang, X.,** Brockdorff, N., Abate-Shen, C., Famham, P., Reinberg, D. (2005). Composition and histone

substrates of polycomb repressive group complexes change during cellular differentiation. *Proc Natl Acad Sci USA* 102:1859-64.

3. **Ouyang, X.,** DeWeese, T.L., Nelson, W.G., Abate-Shen, C.. Loss-of-function of *Nkx3.1* promotes increased oxidative damage in prostate carcinogenesis. (Submitted)
4. **Ouyang, X.**, Banach-Petrosky, W.A., Shen, M.M., Abate-Shen, C. The role of *Nkx3.1* in organ specification of the prostate. (In preparation)

**E. Conclusions**

Several lines of evidence have implicated that *Nkx3.1* is a prostatic-specific tumor suppressor whose loss-of-function represents a critical step in prostate cancer initiation. However, the molecular basis is still largely unknown. Using the *Nkx3.1* mutant mouse model, we have performed a broad exploration using Affymetrix gene expression profiling approach, followed by a validation of several specific genes using real-time PCR and immunohistochemistry. Our findings suggest that *Nkx3.1* is required for prostatic epithelial specificity and its loss-of-function leads to deficient secretory function in prostate, which may contribute to the increased susceptible to carcinogenesis.

Moreover, our findings have revealed that loss-of-function of *Nkx3.1* lead to a loss of anti-oxidative protection in prostate as a consequence of aging, which underlies its role in cancer predisposition.

To explore the role of *Nkx3.1* in advanced stages of prostate cancer progression, comprehensive gene expression profiling has been performed using *Nkx3.1;Pten* double mutant and *Nkx3.1;Pten;p27$^{kip1}$* triple mutant prostatic lesions. We have shown that *Nkx3.1* loss-of-function cooperates with heterozygosity of *p27$^{kip1}$* in promotion of prostate carcinogenesis through up-regulation of *cyclin D1*. Furthermore, our findings of a close association between significant up-regulation of *AP-1* components and androgen-independent prostate cancer indicate that up-regulation/activation of AP-1 transcription factors represent a novel pathway in the transition to androgen-independence.

**F. References**

1. Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., Kim, M., Desai, N., Young, P., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R., Abate-Shen, C., and Shen, M. M. (1999). Roles for Nkx3.1 in prostate development and cancer. *Genes Dev* 13: 966-977.
2. Kim, M. J., Bhatia-Gaur, R., Banach-Petrosky, W. A., Desai, N., Wang, Y., Hayward, S. W., Cunha, G. R., Cardiff, R. D., Shen, M. M., and Abate-Shen, C. (2002). Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis. *Cancer Res* 62: 2999-3004.
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4. Gao, H., Ouyang, X., Banach-Petrosky, W., Borowsky, A. D., Lin, Y., Kim, M., Lee, H., Shih, W. J., Cardiff, R. D., Shen, M. M., and Abate-Shen, C. (2004). A critical role for p27kip1 gene dosage in a mouse model of prostate carcinogenesis. *Proc Natl Acad Sci U S A* 101: 17204-17209.
5. Ouyang, X., DeWeese, T. L., Nelson, W. G., and Abate-Shen, C. Loss-of-function of Nkx3.1 promotes increased oxidative damage in prostate carcinogenesis. *submitted*
6. Kuzmichev, A., Margueron, R., Vaquero, A., Preissner, T. S., Scher, M., Kirmizis, A., Ouyang, X., Brockdorff, N., Abate-Shen, C., Farnham, P., and Reinberg, D. (2005). Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. *Proc Natl Acad Sci U S A* 102: 1859-1864.
7. Abate-Shen, C., Banach-Petrosky, W. A., Sun, X., Economides, K. D., Desai, N., Gregg, J. P., Borowsky, A. D., Cardiff, R. D., and Shen, M. M. (2003). Nkx3.1; Pten mutant mice develop invasive prostate adenocarcinoma and lymph node metastases. *Cancer Res* 63: 3886-3890.

**Appendices**

- Gao, H., ***Ouyang, X.,** Banach-Petrosky, W., Borowsky, A.D., Lin, Y., Kim, M., Lee, H., Sun, X., Shih, W.J., Cardiff, R.D., Shen, M.M., Abate-Shen, C. (2004). A critical role for $p27^{kip1}$ gene dosage in a mouse model of prostate carcinogenesis. *Proc Natl Acad Sci USA* 101:17204-9. **(* co-first author)**
- **Ouyang, X.,** DeWeese, T.L., Nelson, W.G., Abate-Shen, C.. Loss-of-function of *Nkx3.1* promotes increased oxidative damage in prostate carcinogenesis. (Submitted)

# A critical role for $p27^{kip1}$ gene dosage in a mouse model of prostate carcinogenesis

Hui Gao*†‡, Xuesong Ouyang*†‡, Whitney Banach-Petrosky*†‡, Alexander D. Borowsky§, Yong Lin¶‖, Minjung Kim*†**, Hansol Lee*†, Weichung-Joseph Shih¶‖, Robert D. Cardiff§, Michael M. Shen*¶††‡‡, and Cory Abate-Shen*†¶‡‡§§

*Center for Advanced Biotechnology and Medicine, ¶Cancer Institute of New Jersey, and Departments of †Medicine, §§Neuroscience, ††Pediatrics, and ‖Biometrics, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, Piscataway, NJ 08854; and §Center for Comparative Medicine, Department of Pathology and Laboratory Medicine, University of California, Davis, CA 95616

Communicated by Keith R. Yamamoto, University of California, San Francisco, CA, October 18, 2004 (received for review July 30, 2004)

**In human prostate cancer, the frequent down-regulation of $p27^{kip1}$ protein expression is correlated with poor clinical outcome, yet *$p27^{kip1}$* rarely undergoes mutational inactivation. Here, we investigate the consequences of reducing or eliminating *$p27^{kip1}$* function for prostate carcinogenesis in the context of a mouse modeling lacking the *Nkx3.1* homeobox gene and the *Pten* tumor suppressor. Unexpectedly, we find that triple mutant mice heterozygous for a *$p27^{kip1}$* null allele (*$Nkx3.1^{+/-\text{ or }-/-}$; $Pten^{+/-}$; $p27^{+/-}$*) display enhanced prostate carcinogenesis, whereas mice that are homozygous null for *$p27^{kip1}$* (*$Nkx3.1^{+/-\text{ or }-/-}$; $Pten^{+/-}$; $p27^{-/-}$*) show inhibition of cancer progression. Expression profiling reveals that *Cyclin D1* is highly up-regulated in compound *$p27^{kip1}$* heterozygotes, but is down-regulated in the compound *$p27^{kip1}$* homozygous mutants. Using RNA interference in prostate cancer cell lines with distinct *$p27^{kip1}$* gene doses, we show that prostate tumorigenicity depends on levels of *$p27^{kip1}$* and that the consequences of *$p27^{kip1}$* gene dosage can be attributed, in part, to altered levels of Cyclin D1. Our findings suggest that *$p27^{kip1}$* possesses dosage-sensitive positive as well as negative modulatory roles in prostate cancer progression.**

Cyclin D1 | expression profiling | p27 | prostate cancer

Prostate cancer now represents a serious health problem worldwide (1). Despite intense investigations, it has remained a daunting task to elucidate the mechanisms underlying disease progression. However, difficulties in studying the disease in humans can be circumvented by using mouse models, which can provide mechanistic insights into prostate carcinogenesis as well as access to all disease stages (2–5). Indeed, recent studies have led to the generation of several relevant models, including those based on the loss-of-function of genes implicated in human prostate cancer, such as the *NKX3.1* homeobox gene and the *PTEN* tumor suppressor (e.g., refs. 6–9). In our previous work, we found that *Nkx3.1;Pten* compound mutants recapitulate stages of prostate cancer progression, including its prostatic intraepithelial neoplasia (PIN), adenocarcinoma, and metastatic disease (7, 8). Given the predictable time course of disease progression in these mice, as well their phenotypic similarities to human prostate cancer, we reasoned that *Nkx3.1;Pten* compound mutants would provide a useful model to investigate the contributions of other genetic factors for cancer progression.

We have now investigated the role of the cell cycle regulator *$p27^{kip1}$* in prostate carcinogenesis in the context of *Nkx3.1;Pten* compound mutant mice. As a tumor suppressor, *$p27^{kip1}$* is somewhat atypical; although it rarely undergoes mutational inactivation, its frequent down-regulation in human prostate cancer is associated with poor clinical outcome (reviewed in refs. 10 and 11). In mutant mice, homozygosity as well as heterozygosity for a *$p27^{kip1}$* mutant allele can lead to tumorigenesis (12–16), and these tumorigenic phenotypes are exacerbated in collaboration with loss of other genes, such as *Pten* (6). $p27^{kip1}$ was identified as a negative regulator of cell cycle progression through its ability to inhibit Cyclin E-Cdk2 complexes; however, its functions *in vivo* are likely to be considerably more complex (e.g., ref. 17) and are not restricted to cell cycle control (e.g., ref. 18).

In our analyses of *Nkx3.1;Pten;$p27^{kip1}$* triple mutant mice, we have found that mice lacking one wild-type *$p27^{kip1}$* allele display an enhancement of the prostate cancer phenotype, whereas those lacking both alleles display a reduction in carcinogenesis specifically in the prostate. These phenotypic differences are correlated with levels of *Cyclin D1* and tumor growth depends on the levels of *$p27^{kip1}$* and/or *Cyclin D1*. Our study reveals a critical role for *$p27^{kip1}$* gene dosage for prostate carcinogenesis, and underscores the importance of *Cyclin D1* for disease progression.

## Materials and Methods

*Nkx3.1*, *Pten*, and *p27kip1* mutant mice and analyses of their prostate phenotypes have been described (16, 19, 20); note that the *$p27^{kip1}$* mutant allele generates a truncated protein corresponding to the first 51 amino acids of the protein. Tissue recombinants were generated using adult prostatic epithelium from wild-type or mutant mice and mesenchyme from rat embryonic urogenital sinus (7, 21). Statistical analyses of prostate phenotype were performed by using cumulative logistic regression models with histological grade as the response variable, and age and genotype as explanatory variables. Immunohistochemical analyses were performed by using paraffin sections of mouse dorsolateral prostate, as described (7, 8, 21). Antibodies were as follows: Ki67 (NovoCastra, New Castle, U.K., 1:2,000); Akt and p-Akt (Ser-473) (Cell Signaling Technology, Beverly, MA, 1:200 and 1:50, respectively); $p27^{kip1}$ (Transduction Laboratories BD, Lexington, KY, 1:200); Cyclin D1 (Zymed, 1:500 for Western blotting; Santa Cruz Biotechnology, 1:100 for immunohistochemistry); Cdk4 (Sigma, 1:2,000); Cyclin E (Sigma, 1:1,000); Pten (Neomarker, 1:200); and pol II (Covance, 1:500). The Nkx3.1 antibody was published previously (8).

Laser-capture microdissection (LCM; Arcturus, Mountain View, CA, Pixcell IIE) was performed on cryosections of snap-frozen prostate tissues to isolate epithelial cells from wild-type prostate, PIN, or cancer lesions. RNA was prepared by using the PicoPure RNA isolation kit (Arcturus), followed by one round of amplification with the RiboAmp RNA amplification kit (Arcturus). Samples were labeled by using a BioArray High Yield transcript labeling kit (Enzo Life Scientific) and hybridized to Affymetrix GeneChips (MOE430A). For statistical analyses, initial data acquisition and normalization were performed by using Affymetrix MICROARRAY SUITE 5.0 software. For validation

Abbreviations: PIN, prostatic intraepithelial neoplasia; RNAi, RNA interference; LCM, laser-capture microdissection.

‡H.G., X.O., and W.B.-P. contributed equally to this work.

**Present address: Dana–Farber Cancer Center, 44 Binney Street, Boston, MA 02115.

‡‡To whom correspondence may be addressed. E-mail: abate@cabm.rutgers.edu or mshen@cabm.rutgers.edu.





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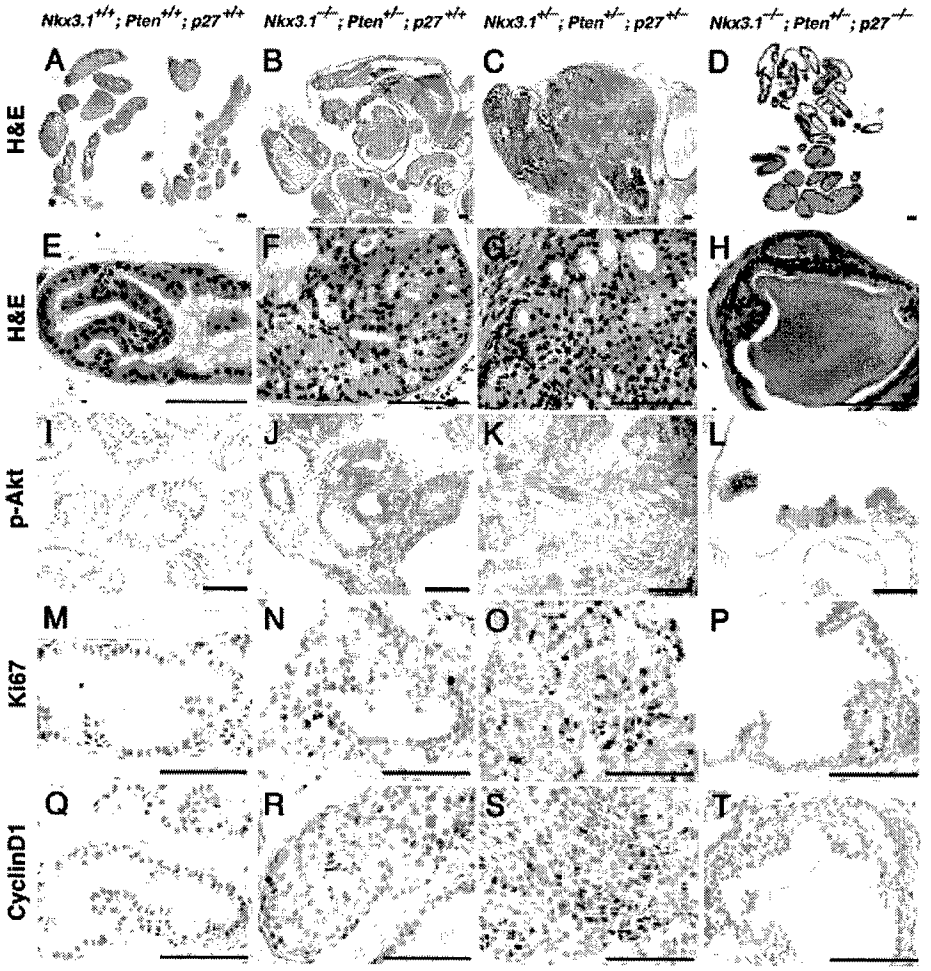


**Fig. 1.** Prostate cancer progression is enhanced in compound mutant mice lacking one $p27^{kip1}$ allele, but inhibited in mice lacking both alleles. Hematoxylin/eosin (*A–H*) or immunohistochemical (*I–T*) staining of paraffin sections from the dorsolateral prostate of mice of the indicated genotypes. (*A* and *E*) Normal prostate histology. (*B* and *F*) High-grade PIN. (*C* and *G*) Adenocarcinoma. (*D* and *H*) Low-grade PIN. (*I–L*) Akt activation in PIN and cancer lesions (also see Fig. 2). (*M–P*) Ki67 staining shows elevated cellular proliferation in compound $p27^{kip1}$ heterozygotes (*O*); also see Fig. 3*B*. (*Q–T*) Elevated expression of Cyclin D1 in compound $p27^{kip1}$ heterozygotes (*S*) (also see Fig. 7). (Scale bars: 100 $\mu$m.)

of gene expression differences, quantitative RT-PCR analyses were performed by using the Mx4000 Multiplex Quantitative PCR system (Stratagene). Expression levels of $p27^{kip1}$ or *Cyclin D1* were evaluated relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH); all validation analyses used at least three independent RNA samples.

RNA interference (RNAi) was performed in prostate epithelial cells made from triple mutant mice by using a retroviral strategy as described (22); infection efficiency was 80% or better, as determined by GFP expression. *Cyclin D1* was overexpressed by retroviral gene transfer by using a vector expressing the mouse gene. Anchorage-independent growth was performed by plating $1 \times 10^3$ cells per 35-mm dish in 0.35% agarose (21). Orthotopic tumor assays were performed by implanting $5 \times 10^5$ cells (10 $\mu$l) unilaterally into the dorsolateral prostate of nude male mice. Following 1 month of growth, tumor weights were determined by comparing the injected and noninjected sides.

Additional methods are provided in *Supporting Material and Methods*, which is published as supporting information on the PNAS web site.

**Table 1. Comparison of $p27^{kip1}$ status in the context of combined *Nkx3.1* and *Pten* loss-of-function**

| Age | Genotype | *n* | Normal/hyperplasia, % | LG-PIN, % | HG-PIN/Cancer, % |
|---|---|---|---|---|---|
| ≤6 mo. | *Nkx3.1*$^{+/-\text{ or }-/-}$; *Pten*$^{+/-}$; $p27^{+/+}$ | 18 | 39 | 28 | 33 |
| | *Nkx3.1*$^{+/-\text{ or }-/-}$; *Pten*$^{+/-}$; $p27^{+/-}$ | 16 | 19 | 43 | 38 |
| | *Nkx3.1*$^{+/-\text{ or }-/-}$; *Pten*$^{+/-}$; $p27^{-/-}$ | 27 | 55 | 26 | 19 |
| >6 mo. | *Nkx3.1*$^{+/-\text{ or }-/-}$; *Pten*$^{+/-}$; $p27^{+/+}$ | 45 | 0 | 27 | 73 |
| | *Nkx3.1*$^{+/-\text{ or }-/-}$; *Pten*$^{+/-}$; $p27^{+/-}$ | 54 | 0 | 19 | 82 |
| | *Nkx3.1*$^{+/-\text{ or }-/-}$; *Pten*$^{+/-}$; $p27^{-/-}$ | 1* | 0 | 0 | 100 |

For clarity, we have grouped the *Nkx3.1* heterozyous and homozygous mutants, because they have similar phenotypes. LG, low grade; HG, high grade.

*Only one mouse of this genotype survived past 6 months.

## Results

**Prostate Cancer Progression Is Enhanced in Compound Mutant Mice Lacking One Wild-Type $p27^{kip1}$ Allele, but Inhibited in Mice Lacking both Alleles.** To investigate the consequences of combined inactivation of *Nkx3.1*, *Pten*, and $p27^{kip1}$ for prostate carcinogenesis, we generated mutant mice carrying targeted germ-line deletions of all three genes. A comprehensive phenotypic analysis was performed on mice from all 18 viable genotypic combinations ($n$ = 375 mice) including histopathological analyses of each prostatic lobe, followed by detailed immunohistochemical analyses of marker expression in representative cases from each genotype (see *Supporting Materials and Methods*). Our discussion herein focuses on the triple mutant mice; the phenotypes of other genotypic combinations are described in Fig. 6 and Tables 2 and 3, which are published as supporting information on the PNAS web site.

We found that compound $p27^{kip1}$ heterozygotes ($Nkx3.1^{+/-}$ or $^{-/-}$;$Pten^{+/-}$;$p27^{+/-}$) displayed a more severe prostate cancer phenotype as compared with *Nkx3.1*;*Pten* compound mutants having two wild-type $p27^{kip1}$ alleles ($Nkx3.1^{+/-}$ or $^{-/-}$;$Pten^{+/-}$;$p27^{+/+}$) (Fig. 1). In particular, the compound $p27^{kip1}$ heterozygotes were 2.4-fold more likely to develop high-grade PIN and/or adenocarcinoma than compound mutants having both wild-type $p27^{kip1}$ alleles ($P$ = 0.0290; Tables 1 and 4, which is published as supporting information on the PNAS web site). Although high-grade PIN/adenocarcinoma occurred earlier and was more prevalent in the compound $p27^{kip1}$ heterozygotes, the histopathology and other features associated with cancer progression were similar to those previously reported for *Nkx3.1*;*Pten* compound mutants (7, 8, 22). These include (*i*) robust activation of phosphorylated Akt; (*ii*) loss of heterozygosity of *Pten* within the lesions; (*iii*) heterogeneous expression of epithelial markers such as E-cadherin; (*iv*) attenuation of the stroma; (*v*) loss of Nkx3.1 protein expression in heterozygotes; (*vi*) metastases to the lymph nodes; and (*vii*) androgen-independence after androgen-ablation (Figs. 1 and 7, which is published as supporting information on the PNAS web site, and data not shown). However, a notable distinction between the prostate phenotype of the compound $p27^{kip1}$ heterozygotes and that of compound mutants having both wild-type $p27^{kip1}$ alleles was a greatly elevated rate of cellular proliferation, as evident by Ki67 immunostaining (Fig. 1 *M–P*). Indeed, the proliferation index within the high-grade PIN/cancer lesions of the compound $p27^{kip1}$ heterozygotes was >20%, compared with <5% in lesions from mice with both $p27^{kip1}$ alleles (Fig. 3*B*).

In striking contrast to our findings for the compound $p27^{kip1}$ heterozygotes, we observed an opposite phenotypic outcome in triple mutant mice with zero wild-type $p27^{kip1}$ alleles (Fig. 1). In particular, the compound $p27^{kip1}$ homozygous mutants ($Nkx3.1^{+/-}$ or $^{-/-}$;$Pten^{+/-}$;$p27^{-/-}$) were 4-fold less likely to develop high-grade PIN and/or adenocarcinoma than the compound $p27^{kip1}$ heterozygotes ($P$ = 0.0074; Tables 1 and 4). Despite the fact that the compound $p27^{kip1}$ homozygous mutants expressed phospho-Akt (Figs. 1*L* and 7), indicative of *Pten* inactivation, the prostate phenotype of the compound $p27^{kip1}$ homozygous mutant mice was relatively mild, rarely progressing beyond low-grade PIN (PIN grades I and II; refs. 22 and 23) and lacking many of the histopathological features associated with the high-grade PIN or cancer phenotype in mutants having one or two wild-type $p27^{kip1}$ alleles. Notably, our findings contrast with a previous study that reported that 100% of $Pten^{+/-}$;$p27^{-/-}$ compound mutant mice develop prostate cancer by 3 months of age (6); however, this previous study antedated the introduction of a consensus nomenclature for prostate cancer phenotypes in mice (23).

Interestingly, other than the prostate, the compound $p27^{kip1}$ homozygous mutants were highly prone to other neoplasms, particularly lymphomas, and they rarely survived past 6 months of age, compared with the compound $p27^{kip1}$ heterozygotes, which often survived to at least 17 months (W.B.-P and C.A.-S, unpublished observations, and ref. 6). Our observations that prostate cancer progression is enhanced by loss of one wild-type $p27^{kip1}$ allele, but inhibited by loss of both alleles, suggest that carcinogenesis of the prostate, but not most other tissues, is sensitive to $p27^{kip1}$ gene dosage.

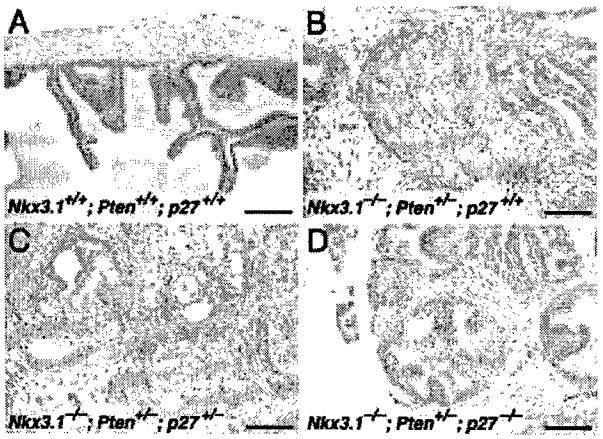


**Fig. 2.** Phenotypic differences among compound $p27^{kip1}$ mutant prostates are retained in tissue recombinants. Hematoxylin/eosin analyses of initial-round tissue recombinants made from prostatic epithelium of the indicated genotypes. (*A*) Normal prostate histology. (*B*) High-grade PIN. (*C*) High-grade PIN with focal areas of microinvasion. (*D*) Low-grade PIN.

**Sensitivity to $p27^{kip1}$ Dosage Is an Intrinsic Property of the Prostatic Epithelium.** To consider the possibility that the relatively mild prostate phenotype of the compound $p27^{kip1}$ homozygous mutants reflected their tendency to succumb to other tumors before having a chance to develop prostate cancer, and to address whether the observed phenotypic differences between the prostatic tissues from mice with two, one, or zero $p27^{kip1}$ alleles were intrinsic to the prostatic epithelium, we performed tissue recombination assays (Fig. 2). We have shown previously that the phenotype of such tissue recombinants closely resembles that of the prostate tissue from which they are derived, and that serial transplantation can result in neoplastic progression, depending on the genotype (7, 24). Thus, we used this approach to evaluate the neoplastic potential of the prostatic epithelium from the triple mutant mice independent of other factors, such as age and/or interactions with the stroma, which might influence the phenotype in the intact mice.

We found that tissue recombinants from compound mutants having two ($Nkx3.1^{+/-}$ or $^{-/-}$;$Pten^{+/-}$;$p27^{+/+}$) or one ($Nkx3.1^{+/-}$ or $^{-/-}$;$Pten^{+/-}$;$p27^{+/-}$) wild-type $p27^{kip1}$ alleles tended to develop high-grade PIN/carcinoma ($n$ = 7/9 and 5/8, respectively), whereas those from mutants lacking any wild-type $p27^{kip1}$ alleles ($Nkx3.1^{+/-}$ or $^{-/-}$;$Pten^{+/-}$;$p27^{-/-}$) rarely developed high-grade PIN or cancer ($n$ = 1/17), and instead displayed low-grade PIN ($n$ = 7/17) or were within normal limits ($n$ = 9/17) (Fig. 2). Importantly, tissue recombinants made from compound $p27^{kip1}$ homozygous mutants also did not progress to more severe phenotypes after serial transplantation ($n$ = 7), in contrast to those having one or two wild-type $p27^{kip1}$ alleles ($n$ = 15) (ref. 7 and data not shown). These tissue recombination studies indicate that phenotypic differences observed among compound mutants having two, one, or zero wild-type $p27^{kip1}$ alleles are stable and intrinsic to the prostatic epithelium, rather than a reflection of the poor survival of the compound $p27^{kip1}$ homozygous mutants.

**Gene Dosage of $p27^{kip1}$ Is Correlated with Levels of Its Expression.** To ascertain whether the phenotypic differences in the prostate of compound mutants having two, one, or zero wild-type $p27^{kip1}$ alleles correlated with the actual levels of $p27^{kip1}$ RNA, we performed

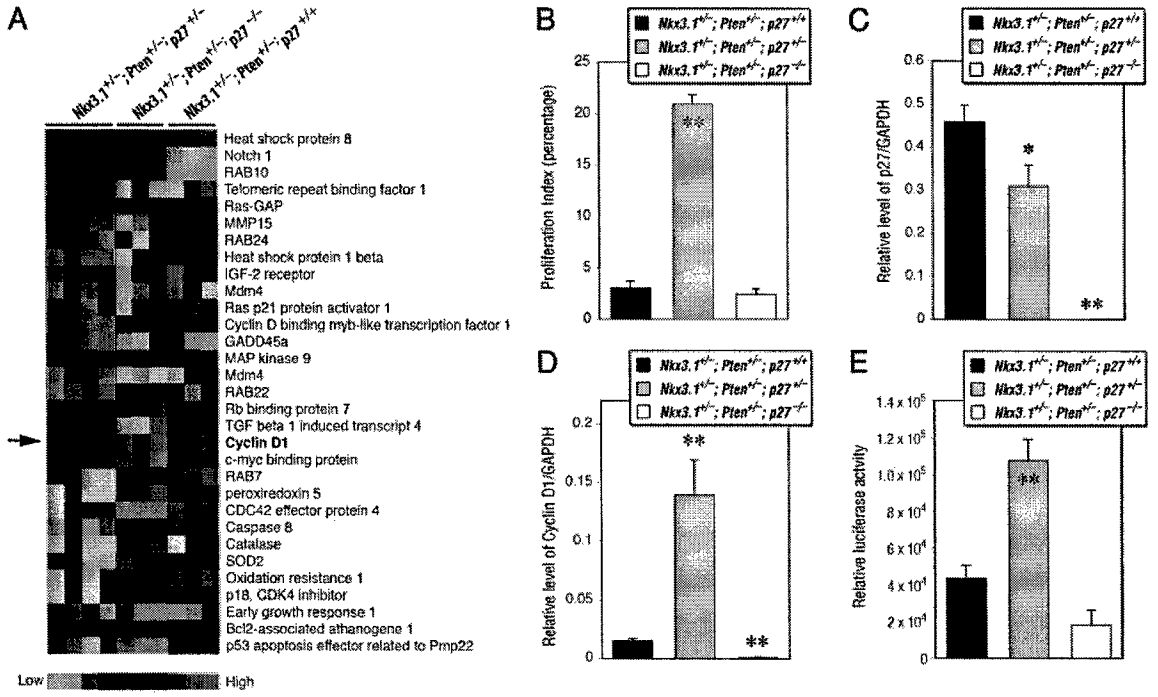


**Fig. 3.** Compound $p27^{kip1}$ heterozygotes display elevated levels of *Cyclin D1*. (*A*) Expression profiling of PIN or cancer lesions was performed by using RNA obtained by LCM of compound mutants of the indicated genotypes. Shown is hierarchical clustering of selected genes of interest. (*B*) Proliferation rate in prostate lesions from triple compound mutants. The percentage of Ki67-positive epithelial cells was determined relative to the total number of epithelial cells, as per Fig. 1 *M–P*. Data represent the average from counting three independent samples (>1,000 cells per sample) for each genotype; error bars indicate the standard error. (*C* and *D*) Real-time PCR analysis of $p27^{kip1}$ (*C*) or *Cyclin D1* (*D*) by using RNA obtained by LCM of prostate epithelial cells from PIN or cancer lesions. Data are expressed relative to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH); analyses were performed in triplicate and error bars show standard error. (*E*) Cyclin D1 promoter activity depends on $p27^{kip1}$ status. Luciferase assays were done in CASP cell lines with varying dosages of $p27^{kip1}$ (see *Materials and Methods*). The Cyclin D1 promoter elements were as mapped previously by Yan and Ziff (33). Shown is their CD1-70 element; similar results were obtained with CD1-1810 and CD1916. *, $P < 0.05$; **, $P < 0.01$.

LCM to isolate prostatic epithelial cells from PIN or cancer lesions of these mice followed by real-time RT-PCR. We found that expression levels of $p27^{kip1}$ RNA were correlated with the number of wild-type $p27^{kip1}$ alleles (Fig. 3*C*). Furthermore, the compound $p27^{kip1}$ heterozygotes also expressed reduced levels of $p27^{kip1}$ protein relative to mice having both $p27^{kip1}$ alleles (Fig. 7), and sequence analysis of genomic DNA isolated from prostate lesions by LCM confirmed that the remaining $p27^{kip1}$ allele in the compound heterozygotes was not mutated (data not shown). Therefore, the differences in $p27^{kip1}$ gene dosage in the compound mutants are directly correlated with levels of $p27^{kip1}$ expression in the PIN and cancer lesions from these mice.

***Cyclin D1* Expression Is Up-Regulated in the Compound $p27^{kip1}$ Heterozygotes but Down-Regulated in the Compound Homozygous Mutants.** To investigate the molecular basis of the observed phenotypic differences between compound mutants with two, one, or zero wild-type $p27^{kip1}$ alleles, we performed gene expression profiling by using RNA isolated from prostate epithelial cells by LCM. We found that the expression profile of the compound $p27^{kip1}$ heterozygotes was distinct from those of mice having either two or zero wild-type $p27^{kip1}$ alleles and included several genes and/or pathways whose deregulation has been implicated in human prostate carcinogenesis (Fig. 3*A* and Table 5, which is published as supporting information on the PNAS web site). Among these deregulated genes was *Cyclin D1*, which was noteworthy given that increased cellular proliferation is a distinguishing feature of the compound $p27^{kip1}$ heterozygotes (Figs. 1 and 3*B*). Real-time RT-PCR analyses confirmed that *Cyclin D1* was significantly up-regulated in cancer lesions from the compound $p27^{kip1}$ heterozygotes, but barely detectable in the compound $p27^{kip1}$ homozygous mutants (Fig. 3*D*), and expression of Cyclin D1 protein was also up-regulated in the compound $p27^{kip1}$ heterozygotes (Figs. 1 *Q–T* and 7). Therefore, the phenotypic differences observed among the triple compound mutants were correlated with differential expression of *Cyclin D1*. Furthermore, by using cell lines that differ in $p27^{kip1}$ gene dosage (see below), we found that *Cyclin D1* promoter activity in transient transfection assays was also dependent on levels of wild-type $p27^{kip1}$ (Fig. 3*E*), suggesting that this differential expression is mediated at the level of *Cyclin D1* transcription.

**Levels of $p27^{kip1}$ and *Cyclin D1* Are Directly Correlated with Prostate Tumorigenicity.** To directly assess the relationship between $p27^{kip1}$ dosage and prostate tumorigenicity, and to evaluate whether *Cyclin D1* is a mediator of this effect, we established a series of prostate cancer cell lines from compound mutant mice having two, one, or zero wild-type $p27^{kip1}$ alleles, which we have named the CASP series (see *Supporting Materials and Methods*). One notable feature of these cell lines is that they are derived from primary tumors rather than metastases, unlike most human prostate cancer cell lines (discussed in ref. 4). The CASP cells retain prostate epithelial properties and form prostatic ducts in cell recombination assays, but also exhibit anchorage-independent growth in soft agar and form tumors when implanted orthotopically into the prostate (Fig. 8, which is published as supporting information on the PNAS web site). Notably, levels of $p27^{kip1}$ protein expression were correlated with its gene dosage, and levels of Cyclin D1 were barely detectable in CASP cells lacking $p27^{kip1}$ (Fig. 8).

To investigate the relationship between $p27^{kip1}$ levels and prostate tumorigenicity, we "knocked-down" the expression of $p27^{kip1}$ in CASP cells by ≈50% by using short-hairpin-mediated RNAi (Fig. 4*A*). The consequences for tumorigenicity were evaluated by assaying anchorage-independent growth in soft agar (Fig. 4*D*), or tumor growth after orthotopic implantation in the prostate (Fig.

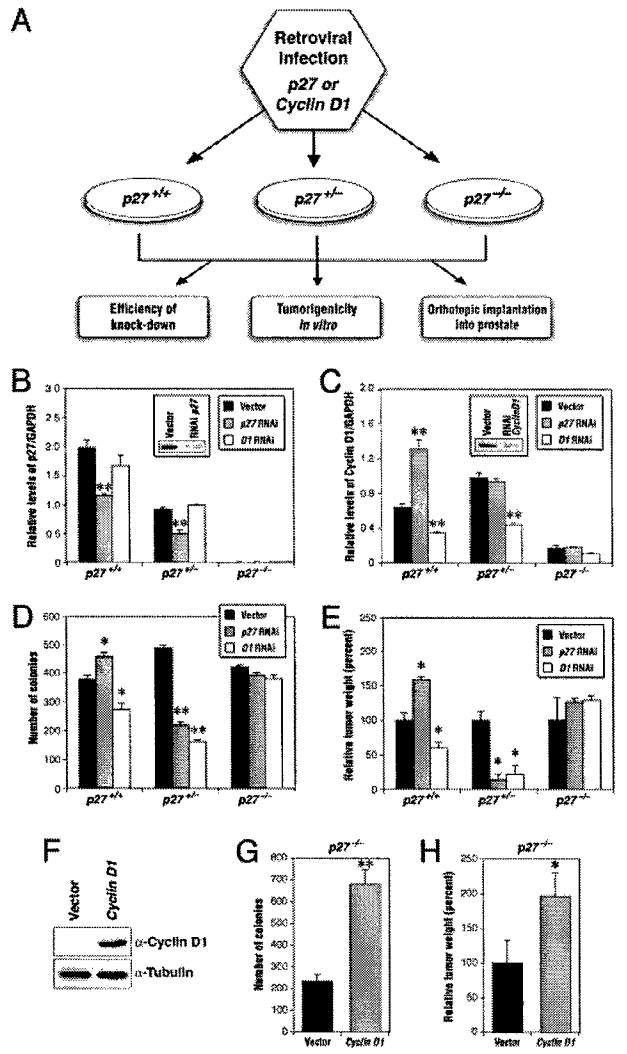


Fig. 4. Levels of $p27^{kip1}$ and *Cyclin D1* are directly correlated with prostate tumorigenicity. (*A–E*) Knock-down of *p27* or *Cyclin D1* in mouse prostate cancer cells. (*A*) Schematic representation of the overall strategy to knock-down of $p27^{kip1}$ or *Cyclin D1* in prostate epithelial cells. CASP cells having two ($p27^{+/+}$), one ($p27^{+/-}$), or zero ($p27^{-/-}$) alleles of $p27^{kip1}$ were infected with retroviruses expressing a control vector, or an RNAi vector specific for $p27^{kip1}$ or *Cyclin D1*. Infected cells were analyzed by real-time PCR and immunoblotting to determine the efficacy of knock-down (*B* and *C*, *Insets*), or were assayed for anchorage-independent growth in soft agar (*D*), or tumor growth after orthotopic implantation into the prostate (*E*). Results represent analyses of five independent experiments each performed in triplicate; error bars indicate standard error. *, $P < 0.05$; **, $P < 0.01$. Similar results were obtained by using two independent cell lines for each $p27^{kip1}$ dose, and with an alternative RNAi sequence for $p27^{kip1}$ and *Cyclin D1* (data not shown). (*F–H*) Forced expression of *Cyclin D1* in mouse prostate cancer cells. (*F*) Western blot showing expression of exogenous Cyclin D1 in prostate epithelial cells with zero wild-type $p27^{kip1}$ alleles ($p27^{-/-}$). (*G*) Anchorage-independent growth. (*H*) Tumor growth after orthotopic implantation into the prostate. Results represent analyses of two independent experiments each performed in triplicate; error bars indicate standard error. *, $P < 0.05$; **, $P < 0.01$.

4*E*). We performed these experiments in CASP cells having two ($p27^{+/+}$), one ($p27^{+/-}$), or zero ($p27^{-/-}$) wild-type $p27^{kip1}$ alleles, with the latter providing an ideal control for RNAi specificity.

Although RNAi for $p27^{kip1}$ had virtually no effect on the $p27^{kip1}$ null cells ($p27^{-/-}$) as expected, in the $p27^{kip1}$ wild-type cells ($p27^{+/+}$) it resulted in ≈50% reduction in the $p27^{kip1}$ levels (Fig. 4*B*). Notably, this partial knock-down of $p27^{kip1}$ in the wild-type cells was coincident with a moderate increase in tumorigenicity *in vitro* and

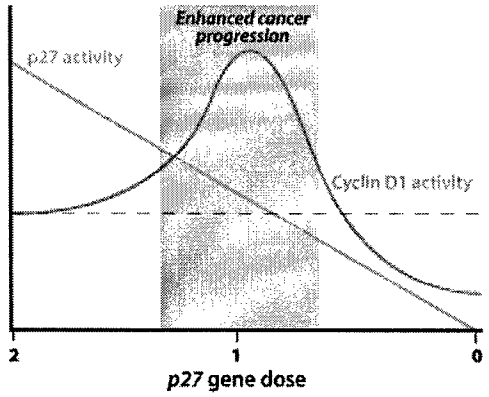


**Fig. 5.** Model for the relationship of $p27^{kip1}$ gene dosage, *Cyclin D1* expression, and prostate tumorigenicity. See *Discussion*.

*in vivo* (Fig. 4 *D* and *E*; $P = 0.0092$ and 0.0038, respectively). In the $p27^{kip1}$ heterozygous cells ($p27^{+/-}$), RNAi of $p27^{kip1}$ also resulted in an ≈50% reduction in $p27^{kip1}$ expression levels to ≈25% of the wild-type levels (Fig. 4*B*), which corresponded to a significant reduction in tumorigenicity both *in vitro* and *in vivo* (Fig. 4 *D* and *E*; $P < 0.0001$ and 0.0027, respectively). These observations are concordant with our histopathological findings in the compound mutant mice and establish a direct relationship between $p27^{kip1}$ gene dosage, $p27^{kip1}$ expression levels, and prostate tumorigenicity.

Finally, to assess the significance of *Cyclin D1* for prostate tumorigenicity, we used RNAi to knock-down its expression in the CASP cells, without affecting expression of $p27^{kip1}$ (Fig. 4*C*). After RNAi for *Cyclin D1*, we observed a significant reduction in tumorigenicity of cells that are wild-type or heterozygous for $p27^{kip1}$ in soft agar assays ($P = 0.0079$ and $P < 0.0001$, respectively), as well as in the orthotopic implantation assay ($P = 0.0228$ and 0.0061, respectively), but virtually no effect on the $p27^{kip1}$ homozygous mutant cells, which already have low levels of Cyclin D1 (Fig. 4 *D* and *E*). Conversely, to address whether restoration of *Cyclin D1* in the $p27^{kip1}$ homozygous mutant cells affected their tumorigenicity, we used retroviral gene transfer to express exogenous *Cyclin D1* in these cells (Fig. 4*F*). This resulted in increased anchorage-independent growth ($P = 0.0002$) and increased tumor growth after orthotopic implantation in the prostate ($P = 0.05$; Fig. 4 *G* and *H*). Taken together, these loss- and gain-of-function studies suggest that the sensitivity of prostate carcinogenesis to $p27^{kip1}$ dosage is mediated, in part, through *Cyclin D1* and demonstrate the significance of *Cyclin D1* for prostate tumorigenicity.

## Discussion

Our analysis of mouse models of prostate cancer has uncovered a critical relationship between gene dosage of $p27^{kip1}$ and prostate tumorigenicity. Surprisingly, a 2-fold reduction in wild-type $p27^{kip1}$ dosage greatly enhances prostate carcinogenesis, whereas its complete loss impedes cancer progression specifically in the prostate. We have demonstrated that these differences in the severity of the cancer phenotype are intrinsic to the prostatic epithelium, and that they are directly related to the levels of wild-type $p27^{kip1}$ expression. Furthermore, we show that these consequences of $p27^{kip1}$ gene dosage for prostate tumorigenesis are at least partially attributed to altered levels of Cyclin D1, underscoring a role for this *Cyclin* gene in prostate carcinogenesis. We propose that a reduction of wild-type $p27^{kip1}$ gene dosage promotes prostate carcinogenesis in part by up-regulating Cyclin D1 expression, whereas elimination of $p27^{kip1}$ leads to down-regulation of Cyclin D1 expression and reduced prostate carcinogenesis (Fig. 5). This finding may explain why $p27^{kip1}$ expression is often down-regulated in prostate cancer, yet is rarely eliminated.

Although this complex relationship between $p27^{kip1}$ gene dosage and prostate carcinogenesis may seem counterintuitive, our findings agree with previous observations reported in studies of mouse models of breast cancer. In particular, Arteaga and colleagues (13) have shown that heterozygosity for $p27^{kip1}$ enhances cancer progression in the context of an *MMTV-c-neu* transgene, whereas nullizygosity for $p27^{kip1}$ inhibits cancer progression. Notably, in their studies, the $p27^{kip1}$ homozygous mutants were found to be deficient for *Cyclin D1/Cdk4* function in mammary epithelial cells (25), comparable to our findings in the prostatic epithelium. Because $p27^{kip1}$ nullizygosity does not generally inhibit carcinogenesis of other tissues, it is intriguing to speculate that the dosage of wild-type $p27^{kip1}$ may be particularly relevant for hormonally regulated cancers. Indeed, a threshold level of $p27^{kip1}$ is required for response to anti-estrogens in the breast (26), whereas $p27^{kip1}$ plays a key role in androgen-stimulated proliferation in the prostate (27).

Previous studies have defined a haploinsufficient role of $p27^{kip1}$ in carcinogenesis in mutant mice, based on the observed effects of $p27^{kip1}$ heterozygosity in enhancing carcinogenesis in a range of tissues. Notably, this previous work demonstrated haploinsufficiency for $p27^{kip1}$ in lung, intestine, and pituitary carcinogenesis, and found that homozygous inactivation leads to increased tumorigenicity relative to heterozygosity (14). However, our demonstration that homozygous deletion of wild-type $p27^{kip1}$ retards cancer progression in the prostate is distinct from a simple model of haploinsufficiency, because we find that heterozygosity and nullizygosity for $p27^{kip1}$ result in opposite phenotypes.

There are few genetic precedents for this relationship of $p27^{kip1}$ gene dosage and phenotypic outcome. One example is provided by studies of *Fgf8* dosage sensitivity in embryonic brain development, where cell survival within the telencephalon is decreased when *Fgf8* expression is either up-regulated or eliminated, but is increased when *Fgf8* expression is down-regulated (28). This unusual type of dosage effect can potentially be explained by a proportionate response of a feedback inhibitory pathway, as in the case of *Fgf8* activity in the brain, or by distinct functional activities that prevail at high versus low protein concentrations, as is likely to be the case for $p27^{kip1}$.

Our data are consistent with a complex mechanistic relationship between $p27^{kip1}$ and *Cyclin D1* that has previously been inferred based on biochemical and genetic analyses. Despite its original identification as a cell cycle inhibitor, $p27^{kip1}$ may also have a positive effect on the cell cycle, because it can interact with Cyclin D1–Cdk4 protein complexes, thereby stabilizing these complexes and promoting progression through the $G_1$ phase of the cell cycle (29). Moreover, $p27^{kip1}$ also has cell cycle-independent functions that may contribute to the need for its residual expression for prostate carcinogenesis. Although our findings indicate that altered levels of *Cyclin D1* represents a principal outcome of varying $p27^{kip1}$ dosage, we cannot exclude the possibility that other functions of $p27^{kip1}$ are also important, such as the recent identification of its role in cell migration (18). Indeed, our expression profiling analyses have revealed that the compound heterozygotes display alterations in expression of Rho family genes (see Table 4).

Our findings highlight a hitherto unappreciated role for *Cyclin D1* in prostate carcinogenesis. However, Cyclin D1 has been reported to be up-regulated in advanced prostate cancer in humans, particularly at stages associated with androgen independence and metastases to bone (30). Although our findings in mutant mice are consistent with the idea that up-regulation of *Cyclin D1* is associated with increased cellular proliferation, it is conceivable that the consequences of its up-regulation in the prostatic epithelium are not solely mediated through its effects on cell cycle progression. Indeed, *Cyclin D1* has been shown to function independently of Cdk4 as a corepressor of androgen receptor (31); this finding may partially explain its particular relevance for hormonally regulated cancers, because *Cyclin D1* can also regulate the transcriptional activity of estrogen receptor (e.g., ref. 32).

In summary, our findings indicate that prostate cancer progression is highly sensitive to wild-type $p27^{kip1}$ expression levels, because a 50% reduction of $p27^{kip1}$ activity due to heterozygous inactivation enhances prostate tumorigenesis, whereas a further 50% reduction (to $\approx$25% of wild-type levels) is sufficient to reverse this effect. Thus, a broad spectrum of functional consequences can be observed over a 4-fold range of $p27^{kip1}$ activity. This narrow dosage window suggests that down-regulation of $p27^{kip1}$ expression levels or activity may represent an effective route for therapeutic intervention, and that our mutant mice may provide a valuable preclinical model for testing these potential therapies.

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# Loss-of-function of *Nkx3.1* promotes increased oxidative damage in prostate carcinogenesis

Xuesong Ouyang[1,3], Theodore L. DeWeese[5,6], William G. Nelson[6,7], and Cory Abate-Shen[1,2,3,4,8]

[1]Center for Advanced Biotechnology and Medicine, [2]The Cancer Institute of New Jersey, Departments of [3]Medicine and [4]Neuroscience, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ

Departments of [5]Radiation Oncology and Molecular Radiation Sciences, [6]Urology, and [7]Oncology, Johns Hopkins University School of Medicine, Baltimore, MA

[8]To whom correspondence should be addressed at: 732-235-5161 (phone), 732-235-5789 (fax), abate@cabm.rutgers.edu

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**Abbreviations**: PIN, Prostatic intraepithelial neoplasia; 8-OhdG, 8-hydroxy-2'-deoxyguanosine; 4HNE 4-hydroxynonenal; *GPx*, glutathione peroxidase *Prdx6* peroxiredoxin 6; Qscn6, sulfyhydryl oxidase Q6; ROS, reactive oxygen species; SOD, superoxide dismutase; LC-MS/MS, liquid chromatography-tandem mass spectroscopy.

**Abstract**

Despite the significance of oxidative damage for carcinogenesis, the molecular mechanisms that lead to increased susceptibility of tissues to oxidative stress are not well understood. We now report a link between loss of protection against oxidative damage and loss-of-function of *Nkx3.1*, a homeobox gene that is known to be required for prostatic epithelial differentiation and suppression of prostate cancer. Using gene expression profiling, we find that *Nkx3.1* mutant mice display de-regulated expression of several anti-oxidant and pro-oxidant enzymes, including glutathione peroxidase 2 and 3 (*GPx2, GPx3*), peroxiredoxin 6 (*Prdx6*), and sulfyhydryl oxidase Q6 (*Qscn6*). Moreover, formation of prostatic intraepithelial neoplasia (PIN) in these mutant mice is associated with increased oxidative damage of DNA, as evident by increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG). We further show that progression to prostate adenocarcinoma, as occurs in compound mutant mice lacking *Nkx3.1* as well as the *Pten* tumor suppressor, is correlated with a further de-regulation of anti-oxidants, including superoxide dismutase (SOD) enzymes, and more profound accumulations of oxidative damage to DNA and protein, the latter manifested by increased levels of 4-hydroxynonenal (4HNE). We propose that the essential role of *Nkx3.1* in maintaining the terminally differentiated state of the prostate epithelium provides protection against oxidative damage and, thereby, suppression of prostate cancer. Thus, our findings provide a molecular link between a gene whose inactivation is known to be involved in prostate carcinogenesis, namely *Nkx3.1*, and oxidative damage of the prostatic epithelium.

## Introduction

The persistent generation of reactive oxygen species (ROS) in cells, which is a byproduct of mitochondrial respiration (and other sources), is an inevitable consequence of aging in aerobic organisms (1-3). At low or moderate intracellular levels, ROS play important roles in signal transduction and regulation of redox-sensitive transcription factors (4, 5). However, elevated levels of ROS, which accrue with aging and/or exposure to dietary or environmental carcinogens, are coincident with an accumulation of genetic alterations that are associated with carcinogenesis (2, 6-8). Among the major risk factors for prostate cancer, for example, are aging and exposure to carcinogens (9). Indeed, age-related increases in free radicals have been associated with increased risk for prostate cancer (10), while sequence variants of a gene encoding a repair enzyme (*hOGG1*) that protects against oxidative damage of DNA leads to increased prostate cancer susceptibility (11). Conversely, anti-oxidants, such as Vitamins E, selenium, lycopene, have been implicated in reducing the risk of prostate cancer (9). However, despite a great deal of attention regarding the relationship between oxidative damage and carcinogenesis, the mechanisms that contribute to increased susceptibility of tissues to oxidative stress are not well understood.

Among the cellular defenses against elevated levels of ROS and accumulation of oxidative damage are anti-oxidant enzymes, which act in concert to provide a coordinated network of protection against ROS accumulation and oxidative damage (4, 8, 12-15). For example, SOD enzymes in mitochondria convert superoxide to hydrogen peroxide, which is then further reduced by the selenium-containing GPx enzymes located in the mitochondria (Gpx1), cytosol (Gpx2), or plasma membrane (Gpx3). Other anti-oxidants

include the peroxiredoxins (Prdx), a ubiquitous family of thiol-containing enzymes which are also major reductants of endogenously produced peroxides. Opposing the actions of these anti-oxidant enzymes are pro-oxidants whose cellular activities result in increased accumulation of ROS. These include the sulfyhydryl oxidase Q6 (Qscn6), which generates hydrogen peroxide as a byproduct of the oxidization of sulfhydryl groups in the course of generating disulfide-containing secreted peptides (16).

Thus, perturbations of the balance in expression and/or function of anti-oxidant and pro-oxidant enzymes combined with exogenous factors (*i.e.,* aging, etc.) that lead to accumulation of ROS may have profound consequences for oxidative damage. Indeed, the relationship between altered protection against ROS and carcinogenesis is highlighted by numerous examples in which anti-oxidant enzymes have been shown to be de-regulated expression in cancer (*e.g.,* (6, 14, 17-19), as well as the consequences of the loss-of-function of anti-oxidant activities for carcinogenesis in mutant mouse models. For example, loss-of-function of *GPx1* and *GPx2* in mutant mice results in increased susceptibility to inflammation and cancer in the intestine (20), while mice lacking either *Prdx1* or *Prdx6* display increased levels of ROS, elevated oxidation damage, and increased propensity for tumor formation (21, 22).

We have been investigating the mechanisms underlying prostate cancer initiation through our analyses of the *Nkx3.1* homeobox gene (23). In previous studies, we have shown that *Nkx3.1* displays restricted expression to the prostatic epithelium and that its inactivation leads to inappropriate prostate epithelial differentiation (24). Moreover, *Nkx3.1* mutant mice develop PIN as a consequence of aging (24-26), which progresses to metastatic adenocarcinoma in compound mutant mice lacking *Nkx3.1* as well as the *Pten*

tumor suppressor (27, 28). In humans, *NKX3.1* is located on a region of chromosome 8p21, which frequently undergoes allelic imbalance in PIN as well as prostate cancer (29-32). Although *NKX3.1* is not mutated in prostate cancer (23, 33, 34), its inactivation by loss of protein expression is a hallmark of cancer progression in humans and mouse models (25, 26, 35-37). These and other lines of evidence support the idea that *Nkx3.1* loss-of-function plays an important role in prostate-specific cancer initiation.

In our gene expression profiling analyses, we have now found that *Nkx3.1* mutant mice display de-regulated expression of several anti-oxidant and pro-oxidant enzymes, which is accompanied by increased oxidative damage of DNA in the aged mice. Furthermore, cancer progression in *Nkx3.1; Pten* compound mutant mice is associated with additional perturbations of anti-oxidant enzymes as well as further accumulations of oxidative damage of DNA and protein. Thus, our findings demonstrate that *Nkx3.1* loss-of-function leads to reduced protection against ROS and increased oxidative damage, which thereby provide a molecular link between a gene that predisposes to prostate cancer and oxidative damage. We propose that the *Nkx3.1* mutant mice will be valuable for studying the relationship between the oxidative damage response and carcinogenesis, as well for pre-clinical studies to test the efficacy of potential anti-oxidants for prostate cancer prevention.

**Materials and Methods**

Generation and analysis of mutant mice harboring null alleles of *Nkx3.1* and/or *Pten* have been described previously (24, 26, 27, 38). To avoid variability due to differences in strain background, gene expression profiling was performed using whole anterior prostate from age-matched (*i.e.,* 15 months) cohorts of *Nkx3.1* homozygous mutants ($Nkx3.1^{-/-}$) or wild-type littermate controls ($Nkx3.1^{+/+}$) on an inbred C57Bl/6J strain background (26). To further minimize variability from individual specimens, prostate tissues from three independent animals were pooled to generate RNA for each array and a minimum of three arrays were probed for the wild-type and mutant mice (thus allowing comparison of a total of nine mice for each). RNA was extracted using Trizol (Invitrogen) and purified using an RNAeasy kit (Qiagen). cDNA was labeled using a BioArray High Yield RNA transcript labeling kit (Enzo Life Scientific) and hybridized to Affymetrix GeneChips (Mu74AV2). For statistical analyses, initial data acquisition and normalization was performed using Affymetrix Microarray Suite 5.0 software followed by an ANOVA test.

Validation of gene expression changes by quantitative RT-PCR was done using an Mx4000 Multiplex Quantitative PCR system (Stratagene). Validation to tissue sections was done by *in situ* hybridization or immunohistochemistry as described (24, 27), depending on the availability of antisera. For Western blot analyses, anterior prostate tissues were snap-frozen on liquid nitrogen and protein extracts were made by sonication in buffer containing 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, 0.1% SDS, 1% deoxycholate (sodium salt), 1% Triton X-100, with freshly added protease inhibitor and phosphatase inhibitor cocktail (Sigma). For *in situ* hybridization sequence-verified

EST clones were purchased from Invitrogen. Antibodies for immunostaining or Western blotting were as follows: Akt and p-Akt (Ser473) (Cell Signaling, 1:200 and 1:50, respectively); Cu/Zn SOD (Upstate, 1:1000); Mn SOD (Upstate, 1:250 for immunohistochmistry; 1:1000 for Western blotting); tubulin (Sigma, 1:1000); Pten (Neomarker, 1:200); 8-OHdG and 4HNE (Japan Institute for the Control of Aging, 1:50 and 1:80, respectively). Antisera against Qscn6 and Nkx3.1 were published previously (16, 27), respectively.

8-OHdG levels were measured by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) as described (39). Briefly, genomic DNA from the whole anterior prostate of *Nkx3.1* wild-type or mutant mice was hydrolyzed to individual nucleosides and a PE Biosystems high-performance liquid chromatography was used to separate hydrolyzed DNA bases. 5-N15–8-oxoG from N15 algal genomic DNA (Spectra Stable Isotopes, Columbia, MD) was used as an internal standard. The detection limit for 8-OHdG with this method is 5 fmol with a small standard deviation due to co-elution with the N15–8-oxoG internal standard. For each sample, at least three independent measurements of dG and 8-OHdG, each with independent calibrations, were performed. All analyses were performed in a blinded fashion.

## Results and Discussion

### Loss-of-function of *Nkx3.1* leads to de-regulated expression of anti- and pro-oxidative enyzmes and increased oxidative damage of DNA.

As a strategy to investigate the mechanisms by which loss-of-function of *Nkx3.1* contributes to formation of PIN, we performed gene expression profiling comparing *Nkx3.1* homozygous null mutants with wild-type littermates as controls. We performed these analyses using aged mice (>12 months), by which time a majority of *Nkx3.1* mutants display PIN (24, 27). Prior to inclusion in the study, we examined the histological phenotype of each experimental mouse to verify the occurrence of PIN (data not shown). We focused on the anterior prostate, which displays the most severe PIN phenotype relative to the other prostatic lobes (24, 27). Gene expression profiling was performed using RNA obtained from the whole anterior prostate; however, the results were validated using RNA obtained by laser capture microdissection of epithelial cells (see below) to verify that the genes of interest were altered in the prostatic epithelial cells, as opposed to stromal or other cellular components present in the whole prostate tissue.

Our analyses revealed a total of 638 genes that were differentially expressed in anterior prostate of the *Nkx3.1* mutants compared with the wild-type mice (see Supplementary data). These included 299 genes that were up-regulated in the mutants and 339 that were down regulated. Interestingly, many of these genes had been reported previously in an analysis of gene expression changes during castration-regeneration of the prostate in *Nkx3.1* mutants, although this previous study was done with a distinct *Nkx3.1* mutant allele and by combining all of the prostatic lobes (40).

We noticed that among the de-regulated genes in our analyses of *Nkx3.1* mutants

was a signature of those known to protect against or to promote oxidative damage (Fig. 1A). Specifically, *Nkx3.1* mutant mice displayed a significant reduction in expression of *GPx2* and *Prdx6* encoding anti-oxidant enzymes and up-regulation of the *GPx3* anti-oxidant and the *Qscn6* pro-oxidant (Fig. 1A). We confirmed these results by real-time RT-PCR using RNA obtained by laser capture microdissection of prostatic epithelium from the anterior prostate of *Nkx3.1* mutant or wild-type mice at 6 or 12 months of age, as well as from the dorsolateral prostate (Fig. 1B, Table 1, and data not shown).

To investigate whether the de-regulated expression of pro- and anti-oxidant enzymes in the prostatic epithelium of the *Nkx3.1* mutants was correlated with oxidative damage in this tissue, we used a quantitative approach to measure levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is a marker of DNA damage (12, 39). These analyses revealed that *Nkx3.1* mutant prostates displayed a significant (~5-fold) increase in the levels of 8-OHdG at 12-months, but not in the younger mice (Fig. 1C). Since the de-regulated expression of anti-oxidant and pro-oxidant enzymes in the *Nkx3.1* mutants precedes the increased accumulation of 8-OHdG, we infer that over time this loss of protection leads to an aberrant accumulation of ROS and ultimately increased oxidative damage. Notably, the observed increase in DNA damage in the *Nkx3.1* mutants is coincident with the onset of PIN phenotypes in these mice (26).

**De-regulated expression of pro-oxidant and anti-oxidant enzymes in the prostatic epithelium of *Nkx3.1* mutant mice.**

To further investigate the relationship between loss-of-function of *Nkx3.1* and de-regulated expression of genes that control ROS levels, we examined the expression of these genes in prostate tissues from *Nkx3.1* wild-type or mutant mice by *in situ*

hybridization, or immunohistochemistry if suitable antisera were available. We focused on the anterior prostate of mice from 4 months to 12 months of age (Fig. 2, Table 1 and data not shown). Note that the wild-type mice (Fig. 2A) display normal prostate histology, whereas *Nkx3.1* mutant mice at 12 months display PIN lesions (Fig. 2B), as defined by histological criteria previously described (41).

We were puzzled by our observation that *GPx2* and *Prdx6* were *down-regulated* in the *Nkx3.1* mutant prostates, whereas *GPx3* (which also encodes an anti-oxidant enzyme) was *up-regulated* (see Fig. 1B). *In situ* hybridization analyses revealed that *Gpx2* and *Prdx6* were expressed throughout the prostatic epithelium of the wild-type mice while their expression was uniformly down-regulated in the prostatic epithelium of *Nkx3.1* mutants (Fig. 2D, E, J, K). In contrast, expression of *Gpx3* was barely detectable in the wild-type prostate, whereas it displayed patchy expression in PIN lesions of the *Nkx3.1* mutants but was not uniformly expressed throughout the epithelium (Fig. 2G, H). Our interpretation is that up-regulation of *Gpx3* may occur in PIN lesions to compensate for the loss of protection by *Gpx2* and/or *Prdx6*.

Notably, the down-regulation of *Gpx2* and *Prdx6* expression occurred in *Nkx3.1* mutant mice as young as 4 months of age (Table 1 and data not shown), which is well before the occurrence of PIN phenotypes in these mice (>8 months (24, 26)). Moreover, since *GPx1* expression was not altered in the *Nkx3.1* mutant mice (Table 1, Fig. 1A), it is unlikely that de-regulated expression of *GPx2* is secondary to other effects of *Nkx3.1*, such as an effect on production of selenium. Thus, our findings suggest that the de-regulated expression of *Gpx2* and *Prdx6* in the mutant mice is likely to be a primary consequence of loss-of-function of *Nkx3.1* rather than secondary to the PIN phenotype.

In addition to the de-regulated expression of genes encoding *anti*-oxidant enzymes, our gene expression profiling data also revealed the up-regulation of a gene encoding the *pro*-oxidant enzyme, *Qscn6* in the *Nkx3.1* mutants. Although not normally expressed at high levels in the wild-type prostate (Fig. 2M), expression of Qscn6 in other tissues is correlated with the demand for disulfide-containing secreted peptides (16). Immunohistochemical staining for Qscn6 revealed its uniform up-regulation throughout the prostatic epithelium of the *Nkx3.1* mutants (Figs. 2N), which was further confirmed by Western blot analyses (Fig. 3). The de-regulated expression of Qscn6 is of particular interest given the known requirement of *Nkx3.1* for appropriate production of prostatic secretory proteins (24). Interestingly, similar to the down-regulation of *Gpx2* and *Prdx6*, up-regulation of Qscn6 occurred in mutant mice as young as 4 months, well before the onset of PIN (data not shown), suggesting that it is also a primary consequence of *Nkx3.1* loss-of-function of rather than secondary to the PIN phenotype.

Taken together, these findings suggest that loss-of-function of *Nkx3.1* perturbs the normal balance of anti-oxidant to pro-oxidant activities in the prostatic epithelium. We infer that this leads to the aberrant accumulation of ROS, which over time leads to increased oxidative damage of DNA and ultimately contributes to formation of PIN.

**Prostate cancer progression is associated with further deficiencies in antioxidant protection**

To further investigate the relationship of de-regulated expression of anti-oxidant and pro-oxidant enzymes for prostate cancer progression, we employed compound mutant mice lacking *Nkx3.1* as well as the *Pten* tumor suppressor gene, which develop high-grade PIN/carcinoma *in situ* by 6 months and adenocarcinoma by 12 months (27,

28). Figure 2C shows an example of the adenocarcinoma phenotype in mice lacking both alleles of *Nkx3.1* and one allele of *Pten* (*Nkx3.1*$^{-/-}$;*Pten*$^{+/-}$). Cancer progression in these mice is coincident with loss of the wild-type allele of *Pten* and up-regulated expression of activated Akt, its major downstream effector (Fig. 3 and (27)).

As expected, expression of *GPx2* and *Pdrx6*, which were uniformly down-regulated in the prostatic epithelium of *Nkx3.1* single mutants, were also barely detectable in the *Nkx3.1; Pten* compound mutants, while expression of Qscn6 continued to be elevated in the *Nkx3.1; Pten* mutants (Fig. 2F, L, O and Fig. 3). However, *Gpx3*, which had been up-regulated in the PIN lesions of the *Nkx3.1* mutants, was not expressed in the *Nkx3.1; Pten* compound mutants (Fig. 2I), suggesting that the compensation provided by *GPx3* in PIN may be lost during progression to adenocarcinoma.

We also observed additional losses of anti-oxidant enzymes in the *Nkx3.1; Pten* compound mutants, including reduced expression of Mn SOD and Cu/Zn SOD (Figs. 2P-R and 3). Unlike *GPx2*, *Prdx6*, and *Qscn6*, Mn SOD and Cu/Zn SOD were not de-regulated in younger mice but were instead altered in expression only in older mice with more progressed PIN or cancer phenotypes (Figs. 2P-R, 3, Table 1 and data not shown). Interestingly, we did not detect reduced levels of RNA for the genes encoding these enzymes in either the *Nkx3.1* single or the *Nkx3.1; Pten* compound mutants, suggesting that the altered expression of these enzymes may occur post-transcriptionally (Fig. 1A and data not shown). Taken together, these findings suggest that progression to adenocarcinoma in these mutant mice is coincident with additional perturbations of anti-oxidant protection, which are unlikely to be a primary consequence of *Nkx3.1* inactivation and more likely to be a consequence of cancer progression.

Accordingly, we asked whether these additional perturbations in protection against oxidative stress observed in adenocarcinoma in the mutant mice were coincident with further increased oxidative damage. Indeed, immunostaining revealed increased levels of 8-OHdG in the cancer lesions of the *Nkx3.1; Pten* compound mutants, relative to the PIN lesions of the *Nkx3.1* single mutants (compare Figs. 2T and U). Moreover, the cancer lesions, but not the PIN lesions, also displayed damage of proteins, as evident by 4HNE, a marker of protein oxidation (compare Fig. 2W and X and Fig. 3) (12). Therefore, the perturbation in the expression of oxidant protection is correlated with increased oxidative damage in cancer progression.

**Conclusions**

Our findings provide new insights regarding the relationship between loss of protection against oxidative stress and prostate carcinogenesis, as well as the mechanisms by which *Nkx3.1* suppresses prostate cancer (Fig. 4). We have shown that loss-of-function of *Nkx3.1* in mutant mice leads to de-regulated expression of several anti-oxidant and pro-oxidant enzymes (*i.e., GPx2, GPx3, Prdx6*, and *Qscn6*) as well as increased oxidative damage of DNA correlated with the formation of PIN in aged mice. We further show that progression to adenocarcinoma in *Nkx3.1; Pten* compound mutants is associated with additional perturbations of the protective response (*i.e.,* loss of SOD enzymes) and more profound consequences for oxidative damage of protein as well as DNA.

Thus, we propose that one of the principal roles of *Nkx3.1* in prostate cancer suppression is to maintain the integrity of the prostatic epithelium by regulating the expression of genes that provide protection against oxidative damage. We infer that

*Nkx3.1* inactivation perturbs the balance of anti-oxidant protection, which sets up a cascade of events that over time leads to an accumulation of ROS, the promotion of oxidative damage of DNA, and ultimately PIN formation (Fig. 4). Intriguingly, the consequences of loss-of-function of *Nkx3.1* for the oxidative damage response overlap with perturbations that are known to occur upon aging in the rodent prostate (42), which further emphasizes the link between aging and oxidative damage (*e.g.*, (1)) and raises the possibility that *Nkx3.1* inactivation may accelerate aging of the prostatic epithelium. Although loss-of-function of *Nkx3.1* is not sufficient for progression to adenocarcinoma (24-26), it acts in collaboration with other genetic factors such as loss-of-function of *Pten* to promote cancer progression (27, 28). It is possible that the observed cooperativity of loss-of-function of *Nkx3.1* and *Pten* for prostate carcinogenesis (27) is reflected, at least in part, through attenuation of protection against oxidative stress, which leads to an acceleration of oxidative damage of DNA and protein (Fig. 4).

Our findings provide support for the idea that defects in the oxidative response pathway occur *early* in prostate carcinogenesis, a notion that is supported by analyses of the expression of anti-oxidant enzymes in PIN versus cancer in the human prostate (*e.g.*, (18, 19)). Furthermore, it has been suggested that perturbations of the oxidative response pathway in human cells as well as rodent models reflect alterations in androgen-signaling (43, 44), which is noteworthy considering that *Nkx3.1* expression is known to be dependent on androgens in both the human and mouse prostate (45-47).

Given that the functions of *NKX3.1* are highly restricted to the prostate and that its loss-of-function occurs with high frequency at early stages of prostate cancer (reviewed in (23)), it seems plausible that the sensitivity of the prostatic epithelium to oxidative

stress (9) reflects, at least in part, a requirement for *NKX3.1*. In other words, as a consequence of defective protection against oxidative stress that occurs following *NKX3.1* inactivation, the prostatic epithelium would tend to be more vulnerable to ROS that accumulate upon aging and/or exposure to carcinogens. This model is consistent with many of the known properties of *NKX3.1*, including the frequent deletion of at least one allele in prostate cancer, its known function for maintaining prostatic epithelial differentiation, and the age-dependent consequences of its loss-of-function for prostate carcinogenesis (reviewed in (23)).

In conclusion, our findings implicate *Nkx3.1* mutant mice as a valuable and unique model to study the relationship between oxidative damage and cancer progression, as well as the potential role of *Nkx3.1* as a key mediator of these events in the prostatic epithelium. One implication of our study is that men with loss of function of *NKX3.1* would be more likely to benefit from dietary or pharmacological interventions with anti-oxidants and that such treatments would be optimal if administered early in neoplastic transformation. This idea, and others, can be tested in the *Nkx3.1* mutant mice, which are likely to be valuable for pre-clinical studies aimed at investigating the benefits of anti-oxidant treatments and the mechanisms by which they are effective.

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**Figure Legends**

**Figure 1. Loss-of-function of *Nkx3.1* results in deregulation of protection against oxidative damage**

(A) Hierarchical clustering of selected gene expression differences between the anterior prostates of wild-type (*Nkx3.1*$^{+/+}$) and *Nkx3.1* mutant (*Nkx3.1*$^{-/-}$) mice. Shown are selected genes involved in protection against or promotion of oxidative damage. A complete list of gene expression differences is provided in the supplementary data. Red indicates up-regulated genes; green indicates down-regulated genes.

(B) Real-time RT-PCR analysis showing the relative expression of *Gpx2*, *Gpx3*, *Prdx6*, and *Qscn6* (as indicated) in RNA made from anterior prostates from wild-type (*Nkx3.1*$^{+/+}$) and *Nkx3.1* mutant (*Nkx3.1*$^{-/-}$) mice (n=6 per group). Data are expressed relative to expression of GADPH. Each data point represents the relative expression of RNA prepared from independent prostate tissues; the bars indicate the average value from the 6 samples.

(C) Relative levels of 8-OHdG in genomic DNA from age-matched wild-type (*Nkx3.1*$^{+/+}$) and *Nkx3.1* mutant (*Nkx3.1*$^{-/-}$) mice. The data are expressed as value of 8-OHdG measured by LC-MS/MS. Three independent experiments were performed. Shown is one representative experiment performed using 3 DNA samples for each group; each of the DNA samples were measured in triplicate in blinded fashion. The standard errors for each sample are indicated.

**Figure 2. Aberrant expression of anti-oxidant and pro-oxidant genes in PIN and prostate cancer in mutant mice.** Histological analyses of the expression of anti-oxidant

and pro-oxidant enzymes in the anterior prostate of wild-type (*Nkx3.1*$^{+/+}$), *Nkx3.1* mutant (*Nkx3.1*$^{-/-}$), and *Nkx3.1; Pten* compound mutant (*Nkx3.1*$^{-/-}$;*Pten*$^{+/-}$) mice at 12 months.

(A-C) Hematoxylin and eosin (H&E) staining show examples of normal histology in the wild-type prostate (A), PIN in the *Nkx3.1* mutant prostate (B), and adenocarcinoma in the *Nkx3.1; Pten* compound mutant prostate (C).

(D-L) *In situ* hybridization showing wide-spread expression of *Gpx2* and *Prdx6* in the epithelium of the wild-type prostate (D, J), while expression is greatly reduced in the PIN and cancer lesions of the *Nkx3.1* mutant prostate (E, K) and *Nkx3.1; Pten* compound mutant prostate (F, L), respectively. In contrast, *Gpx3* expression is virtually absent in the epithelium of the wild-type prostate (G), while it is expressed in PIN lesions of the *Nkx3.1* mutant (H) but not in cancer lesions of *Nkx3.1; Pten* compound mutant (I).

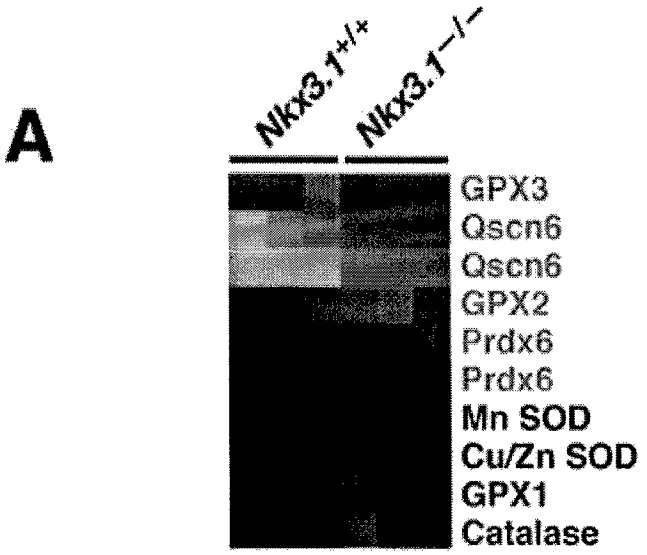
(M-O) Immunohistochemical staining of Qscn6 shows low level expression of this pro-oxidant enzyme in the wild-type prostate (M), while expression is elevated in PIN and cancer regions of *Nkx3.1* mutant (N) and *Nkx3.1; Pten* compound mutants (O).

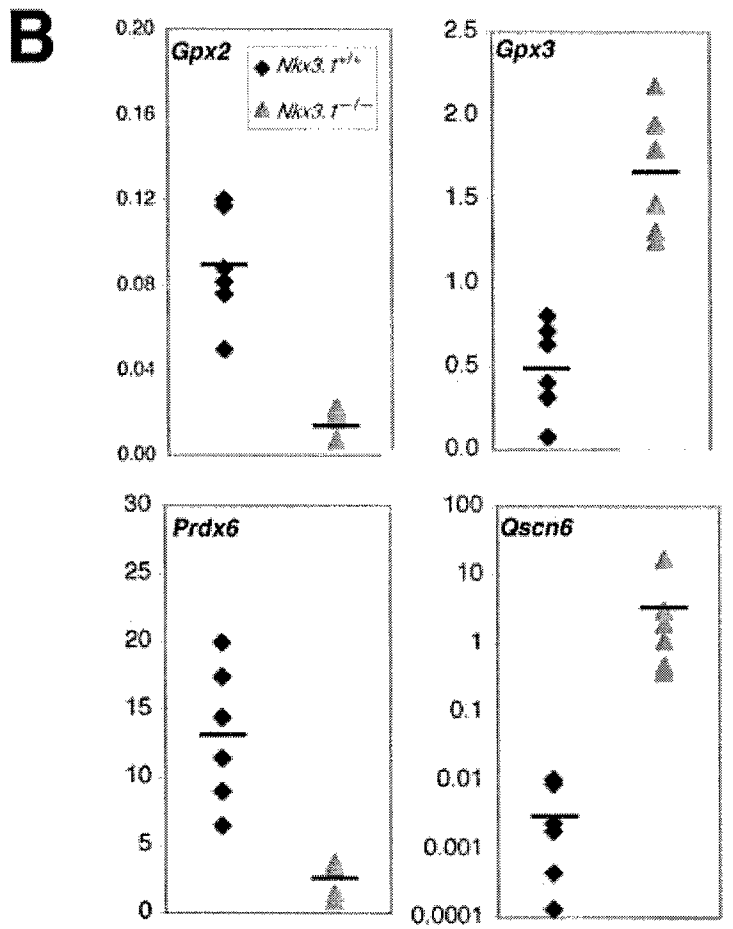
(P-R) Immunohistochemical staining shows slightly reduced expression of Mn SOD in PIN (Q) and significantly reduced expression in cancer (R).

(S-X) Immunohistochemical staining for 8-OHdG and 4HNE shows low-level expression in wild-type prostate (S, V). Expression of 8-OHdG is increased in PIN (T) and further elevated in cancer (U). In contrast, 4HNE is up-regulated in cancer lesions (W, X).

**Figure 3. Deregulated expression of anti-oxidant and pro-oxidant enzymes in PIN and prostate cancer in mouse.** Western blot analyses were done using protein extracts (20 μg/lane) made from anterior prostate of wild-type (*Nkx3.1*$^{+/+}$), *Nkx3.1* mutant (*Nkx3.1*$^{-/-}$), and *Nkx3.1; Pten* compound mutant (*Nkx3.1*$^{-/-}$*;Pten*$^{+/-}$) mice at 12 months. Western blots were probed with the indicated antibodies; Akt and Tubulin are used as an internal control for protein loading. Note that p-Akt is a marker of cancer progression and is up-regulated in the tissues lacking both Nkx3.1 and Pten.

**Figure 4. Model to explain the relationship of loss of protection against oxidative damage and prostate cancer progression.** The model is discussed in the text.


A
Nkx3.1+/+
Nkx3.1−/−
GPX3
Qscn6
Qscn6
GPX2
Prdx6
Prdx6
Mn SOD
Cu/Zn SOD
GPX1
Catalase


B
Gpx2
Nkx3.1+/+
Nkx3.1−/−
0.20
0.16
0.12
0.08
0.04
0.00
Gpx3
2.5
2.0
1.5
1.0
0.5
0.0
Prdx6
30
25
20
15
10
5
0
Qscn6
100
10
1
0.1
0.01
0.001
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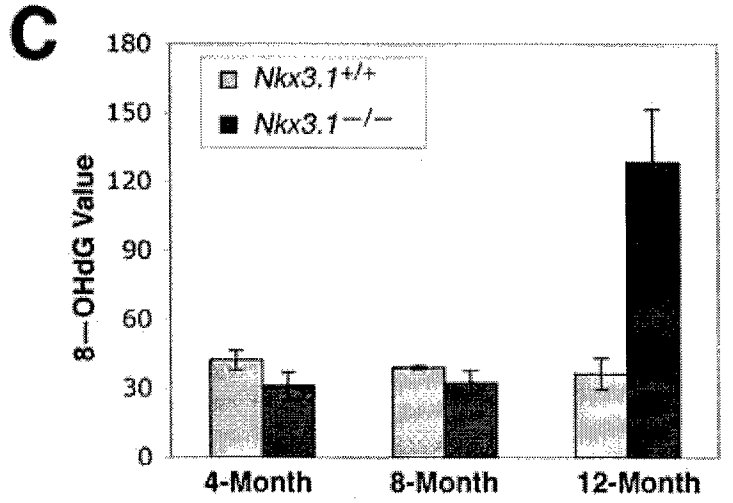
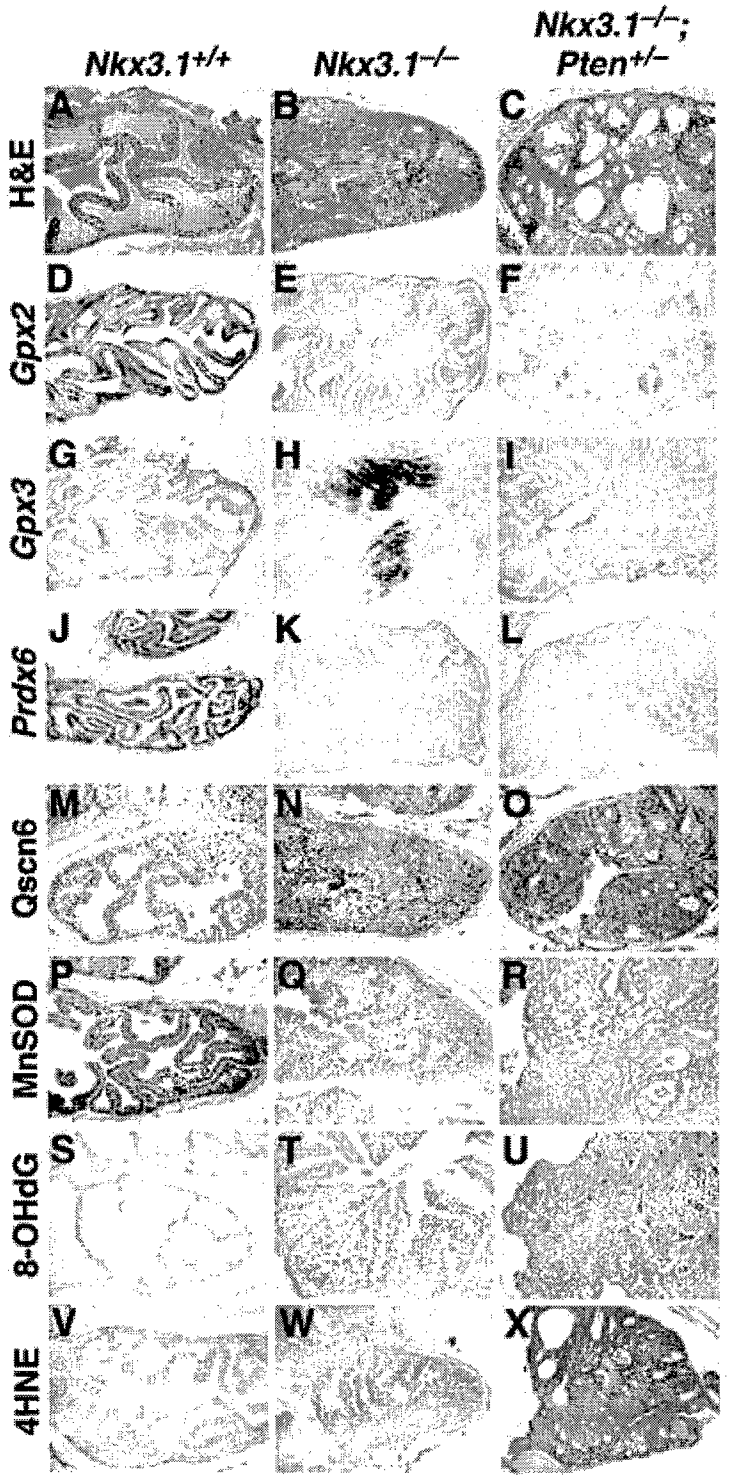

C
Nkx3.1+/+
Nkx3.1−/−
8—OHdG Value
180
150
120
90
60
30
0
4-Month
8-Month
12-Month


Nkx3.1+/+
Nkx3.1−/−
Nkx3.1−/−; Pten+/−
H&E
Gpx2
Gpx3
Prdx6
Qscn6
MnSOD
8-OHdG
4HNE
A
B
C
D
E
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O
P
Q
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S
T
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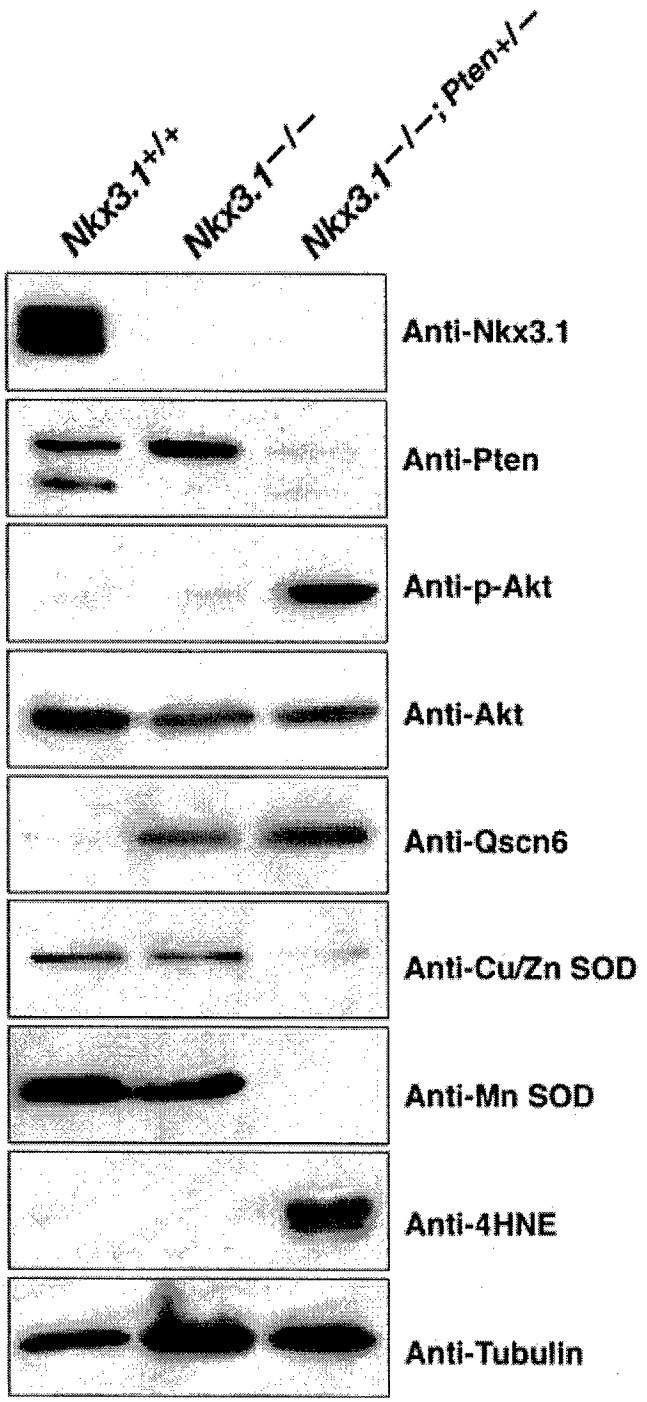
W
X


Nkx3.1+/+
Nkx3.1−/−
Nkx3.1−/−;Pten+/−
Anti-Nkx3.1
Anti-Pten
Anti-p-Akt
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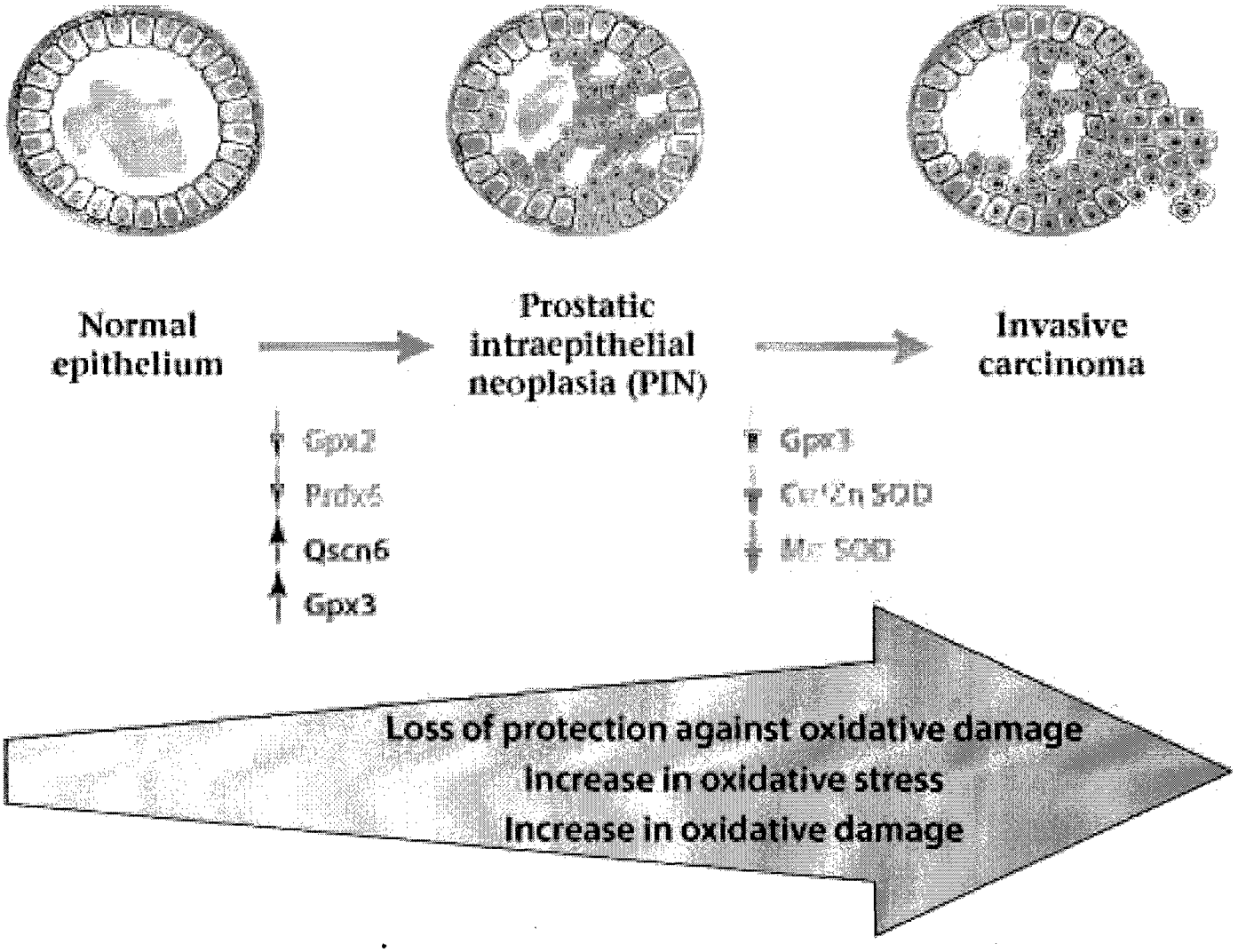
Anti-Cu/Zn SOD
Anti-Mn SOD
Anti-4HNE
Anti-Tubulin


Normal epithelium
Prostatic intraepithelial neoplasia (PIN)
Invasive carcinoma
Gpx2
Prdx6
Qscn6
Gpx3
Gpx3
Cu/Zn SOD
Mn SOD
Loss of protection against oxidative damage
Increase in oxidative stress
Increase in oxidative damage

| Table 1: Summary of changes in anti-oxidant in prostate cancer in mutant mice | | |
|---|---|---|
| **Enzyme** | **Description** | **Expression in normal and mutant mouse prostate** |
| Gpx1 (mitochondrial) | Selenium-dependent anti-oxidant; catalyse the reduction of hydrogen peroxide to oxygen and water | Expressed in wild-type prostatic epithelium (all ages); no change in PIN or cancer in the mutant mice. |
| Gpx2 (cytsolic, epithelial-specific) | | Expressed in prostatic epithelium; uniformly reduced by 4-months in *Nkx3.1* mutant; barely detectable by 12-month in *Nkx3.1* mutants or in cancer lesions of *Nkx3.1; Pten* mutants. |
| Gpx3 (plasma type, secreted) | | Barely detectable in normal prostatic epithelium; up-regulated PIN in *Nkx3.1* mutants from 7-12-months; not expressed in cancer lesions of *Nkx3.1; Pten* mutants. |
| Prdx6 (cystolic) | Thiol-containing anti-oxidant; reduces hydrogen peroxide and alkyl hydroperoxide to water and alcohol | Expressed in wild-type prostatic epithelium (all ages); uniformly reduced in Nkx3.1 mutants by 4-months; barely detectable by 12-month in *Nkx3.1* mutants or in cancer lesions of *Nkx3.1; Pten* mutants |
| MnSOD (mitochondrial) CU/Zn SOD (cytosolic) | Converts superoxide to hydrogen peroxide. | Uniformly expressed in normal prostatic epithelium; slight reduced in PIN in *Nkx3.1* mutants; significantly reduced protein expression in cancer lesions of the *Nkx3.1; Pten* mutants |